EXPERIMENTAL CELL RESEARCH

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ADENOSINETRIPHOSPHATASE ACTIVITY OF RAT LIVER MITOCHONDRIA FOLLOWING *IN SITU* ANOXIA[1]

H. A. LEON, S. F. COOK and J. P. HANNON[2]

Department of Physiology, University of California, School of Medicine, Berkeley, Calif., U.S.A.

Received July 4, 1957

It has previously been noted that *in situ* anoxia causes a rapid linear decrease in the phosphorylating ability of rat liver mitochondria [4]. The part played by ATPase[3] in this decrease has been investigated, and two important points have emerged: (*a*) ionic conditions are of great significance in anoxia and (*b*) information as to mitochondrial organization can be obtained from studies of ATPase activity caused by anoxia.

METHODS

Rat liver mitochondria were obtained as described previously [4] and in all cases were prepared in 0.25 *M* sucrose. In all preparations the ATPase assay media contained the following concentrations: 0.01 *M* sodium–barbital buffer (pH 7.4), 0.002 *M* K-ATP (pH 7.4), and 0.0015 *M* of the chloride salt of either calcium or magnesium. Sucrose was added in varying quantities depending on the situation. The final volume was 1.5 ml in all cases and included 0.2 ml of the mitochondrial preparation.

The incubation was carried out for 15 minutes at 37.5°C in $\frac{3}{8} \times 4$ inch test tubes that had been thermoequilibrated for 2 minutes before addition of the mitochondria. A glass rod placed in the tube served to cause agitation due to the action of the shaking apparatus. The reaction was stopped with 1.0 ml of 12 per cent trichloroacetic acid. Inorganic phosphate was determined by the method of Taussky and Shorr [17], with modification for use with tissues.

Blanks on mitochondrial inorganic phosphate and thermal release of phosphate from ATP were taken into account. The results were then expressed as μ moles of PO_4 released from ATP per mg of nitrogen per 15 minutes.

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[1] This investigation was carried out under Service Grant C-2303 between the U.S. Health Service and the University of California.

[2] Present address: Department of Biochemistry, Arctic Aeromedical Laboratory, A.P.O. 731, Seattle, Wash., U.S.A.

[3] Abbreviations: ATP, adenosine triphosphate; ATPase, adenosinetriphosphatase; (Ca)ATPase, (Mg)ATPase, ATPase activity in the presence of calcium and magnesium, respectively.

RESULTS

Sucrose tonicity and ATPase.—In view of the previously observed influence exerted by osmolarity on ATPase [5, 8], a preliminary study was undertaken to determine the proper composition of the final assay media with respect

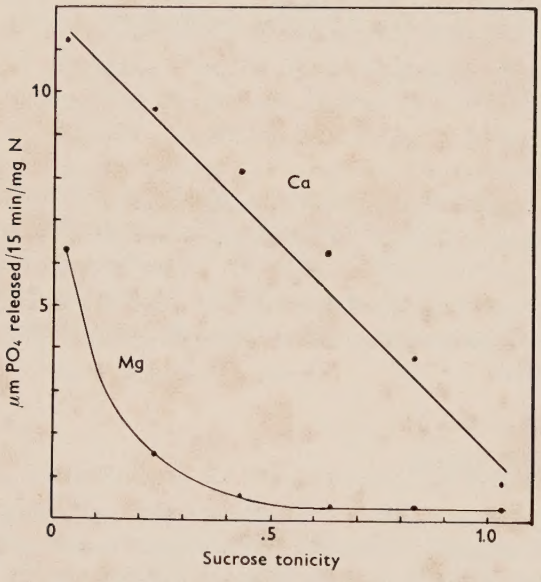

Fig. 1.—Effects of sucrose tonicity on calcium and magnesium activated ATPase of non-anoxic rat liver mitochondria. *Ordinate*, μ moles of PO_4 released from ATP per 15 minutes per mg *N*; *abscissa*, sucrose molarity. See methods section for exact composition of media.

to sucrose tonicity. As a result it was found, as may be seen in Fig. 1, that (Mg)ATPase follows an obviously non-linear decline with increasing sucrose molarity. (Ca)ATPase appears nearly linear in its decline. Although both curves were obtained with mitochondria from a single animal, the experiment is reproducible. The only point of variation is a slight difference in the absolute magnitude of ATPase activity for the different preparations.

ATPase of mitochondria following in situ anoxia.—Since ATPase activity varies with the tonicity of the assay media, determinations on anoxic tissue were made at two tonicities. Both (Mg)ATPase and (Ca)ATPase were determined in 0.033 *M* sucrose which normally gives maximal values as indicated in Fig. 1. Respectively, these are curves *A* and *B* of Fig. 2. For minimal activity, 1.033 *M* sucrose was arbitrarily chosen for (Ca)ATPase (curve *D*, Fig. 2) and for (Mg)ATPase, 0.633 *M* sucrose was employed (curve *C*, Fig. 2). In this respect it is significant that Witter *et al.* [20] found optimum concentrations for the isolation of mitochondria in the range of 0.4 to 0.6 *M* sucrose.

The patterns of the curves are similar to those observed as a result of mitochondrial ageing [20] except that little or no latent period is observed with (Mg)ATPase. This may be due to the presence of intracellular calcium during the anoxic interval. (Ca)ATPase has a higher initial activity which does not appear to increase significantly with increasing anoxic periods.

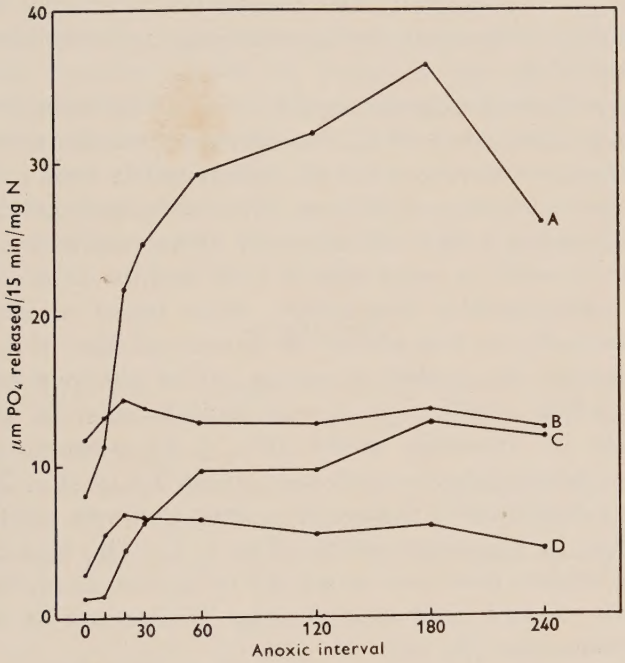

Fig. 2.—Effect of anoxia on the subsequent ATPase activity of liver mitochondria. Each point represents the average value obtained from 5 to 8 animals. *Ordinate*, μmoles of PO_4 released from ATP per 15 minutes per mg *N*; *abscissa*, anoxic interval in minutes prior to measurement. Curve *A*, Mg^{++} activated, hypotonic preparation; curve *B*, Ca^{++} activated hypotonic preparation; curve *C*, Mg^{++} activated hypertonic preparation; and curve *D*, Ca^{++} activated hypertonic preparation. Exact composition of these media described in methods section.

DISCUSSION

According to the results of some investigators [2, 3, 7, 10, 12, 14, 15, 18], the mitochondrion may be viewed as an enzyme complex surrounded by a semi-permeable membrane. Thus, it is endowed with the characteristics of an osmometer [18]. It has also been established that certain diffusion gradients existing in the normal mitochondrion are eliminated coincident with swelling [12]. These gradients, as well as the swollen state, are at least partially reversible. Permeability of the membrane therefore appears de-

pendent on the degree of swelling of the mitochondrion, which, in turn, bears an inverse relationship to the tonicity of the suspending media [5]. Furthermore, the alterations in response to tonicity caused by the presence of either Ca^{++} or Mg^{++} are identical for both ATPase as shown in Fig. 1 and for the relative number of swollen mitochondria as shown by Harman and Kityakara [5]. Consequently, the dependence of ATPase activity on the osmotic properties of the assay media establishes permeability as a limiting factor in this process.

A similar hypothesis is suggested by the results of Kaltenbach and Harman [8] who found that the activity of ATPase showed a much greater dependence on the substrate concentration when the mitochondria were caused to swell. Furthermore, the localization of ATPase *within* the mitochondria by Siekevitz and Potter [15] makes control of its activity by the membrane a probability.

With the above points in mind then, it is felt that the difference in ATPase activity of a mitochondrial preparation, when tested in widely different tonicities, is primarily an indicator of the functional state of the membrane.

If these principles are applied to anoxia, which has long been known to cause mitochondrial swelling [1], it may be stated that the rapid increase in (Mg)ATPase in hypotonic media (Fig. 2, *A*) indicates a progressive damage to the mitochondrial membrane caused by anoxia. However, this damage is, to a considerable degree, reversible as shown by the low values for (Mg)ATPase in hypertonic media (Fig. 2, *C*). The inability to restore completely the initially low levels of activity by hypertonic media at the later anoxic intervals shows a permanent damage. This could be due to factors other than permeability [18, 16].

Although the activity of (Ca)ATPase is higher than (Mg)ATPase in the initial states of anoxia this cannot be taken as evidence that there are two distinct *species* of enzymes, one activated by Mg^{++} and the other by Ca^{++}. There are numerous reports of the deleterious effects of calcium as such on mitochondria such as swelling [6]. Therefore, the initially higher rate of ATPase in the presence of calcium in both Figs. 1 and 2 could be accounted for by damage inflicted on the mitochondria by calcium. This generalized damage could then allow the small amounts of endogenous mitochondrial magnesium [15] to activate ATPase. Further support to this view is given by the substantially unvarying level of (Ca)ATPase as shown in Fig. 2, curves *B* and *D*. Thus, a small quantity of residual endogenous magnesium could activate low but relatively constant amounts of ATPase in calcium damaged mitochondria so that the observed activity would not be altered by anoxia to a great degree. This view is substantiated by the recent work of

Myers and Slater [9] who found four distinct ATPases in mitochondria, none of which were activated further by the addition of calcium to aged preparations. However, one ATPase with pH optimum at 6.3 was activated by calcium in fresh preparations. It is probable then that this pH 6.3 ATPase of Myers and Slater is the same as that illustrated in Fig. 1 where calcium is present.

Regardless of the mechanism, the apparently increased (Ca)ATPase activity in anoxic states becomes significant, since calcium has been observed to enter into cells under conditions of depressed metabolism such as trauma [13]. Magnesium, which counteracts most of the deleterious effects of calcium *in vitro* [6], has been observed to leave the intracellular space under much the same conditons, e.g., shock [19]. Any tendency for calcium to accelerate tissue enzyme damage would therefore become aggravated by losses of magnesium.

SUMMARY

1. A method based upon changes in tonicity was devised which localized ATPase within the mitochondrion and which defines the functional state of the mitochondrial membrane.

2. A comparison between calcium and magnesium as mitochondrial ATPase activators with respect to anoxia showed that the normal activator is magnesium, and calcium is effective only indirectly through injury.

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INCORPORATION OF S^{35}-METHIONINE IN THE CELL FRACTIONS OF SEA URCHIN EGGS AND EMBRYOS[1]

E. NAKANO[2] and A. MONROY

Laboratory of Comparative Anatomy, University of Palermo, Italy

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A METHOD has been worked out recently by the present authors [15] which allows highly efficient incorporation of radioactive isotopes in sea urchin eggs. This method takes advantage of the ability of the mature eggs to take up labelled compounds injected into the body cavity of the adult female. In this way a base-line can be established for investigating the distribution of the isotope among the different components of the unfertilized egg. Upon initiation of development, the change in this distribution can be followed at close time intervals; this is of special importance during cleavage stages, i.e. during the first few hours following fertilization. On the contrary, if incorporation is accomplished by incubating the eggs in sea water containing the labelled compound, a number of shortcomings are met with. Among these the most serious ones seem to be (*a*) the aging of the unfertilized egg during the incubation period, and (*b*) the variations in permeability to the given compound which different stages may exhibit.

By using the above method as a tool for the study of the changes in activity of some cell components during development of the sea urchin embryo, some results have been obtained which will be reported here.

EXPERIMENTAL

Eggs of *Paracentrotus lividus* have been used in the present experiments. As described previously [15] 10 to 20 μC of S^{35}-DL-methionine (in methionine carrier at the final concentration of 100 μC/100 μM/ml) were injected into the body cavity of freshly collected adult females and the animals were then kept at the room temperature (16–19°C) in a moist chamber for four hours. The animals were cut open and the eggs were collected by shaking the ovaries in sea water. The eggs were then filtered through bolting silk, washed three times with sea water and collected by low speed centrifugation. From the final egg suspension a batch was collected and

[1] This investigation has been supported by a grant from Consiglio Nazionale delle Ricerche.

[2] Permanent address: Biological Institute, Nagoya University, Japan. Aided by a grant from the Rockefeller Foundation.

used unfertilized, while the remaining part was fertilized and then subdivided into several portions which were allowed to develop under gentle shaking for the desired length of time. The jelly coat was removed by treating the eggs with acidified sea water.

Before homogenization, the jelly-free eggs or the embryos were packed at the bottom of a centrifuge tube and then suspended in and homogenized with 3.0 ml of 0.3 *M* sucrose in 0.1 *M* citrate buffer at pH 6.3. The homogenate (called "total homogenate") was centrifuged in the cold at 700 g for 10 minutes and the sediment was discarded, while the supernatant ("supernatant first") was centrifuged again at 10,000 *g* for 20 minutes. The sedimented particulate fraction ("particulate fraction") was resuspended in the sucrose solution and centrifuged at 24,000 *g* for 20 minutes. The supernatant ("supernatant second") was also submitted to such a centrifugation. The final sedimented particulate fraction was carefully collected and resuspended in about 1 ml of sucrose and used for the further analyses. Finally, an aliquot of the supernatant second was precipitated with trichloroacetic acid at the final concentration of 10 per cent. After removal of the precipitate, the supernatant ("TCA-soluble fraction") was also used for analysis.

0.1 ml samples were used for analysis. For the measurements of radioactivity the samples were spread uniformly and dried in a thin layer on aluminum planchets. The activity was measured with a thin end-window Geiger–Müller counter and an automatic EKCO scaler. The samples for the determination of total nitrogen of each fraction were combusted and the nitrogen estimated by direct Nesslerization. All determinations were run in duplicate.

RESULTS

Fertilization and Early Development

The unfertilized egg.—The distribution of the isotope between the particulate and the TCA-soluble fractions in unfertilized eggs is given in Table I. Here are also shown the data from three parallel control experiments in which the same batches of eggs were allowed to stand at the room temperature for a period of six hours in the same conditions under which the fertilized eggs were allowed to develop. The data of Table I should also be compared with those of Table III.

The data show first of all that in the unfertilized egg the isotope is stored almost entirely in the TCA-soluble fraction. Upon aging, a slight decrease of the activity of this fraction takes place, while a slight parallel increase of the activity of the particulate fraction occurs.

Changes at fertilization.—In four experiments the changes taking place in the distribution of the isotope in the particulate and in the TCA-soluble fractions in eggs collected 15 minutes after fertilization were investigated. The data are given in Table II.

It appears from these data that 15 minutes after fertilization a slight

TABLE I. *Specific activity (counts/min/1 mg N) in the total homogenate, particulate and TCA-soluble fraction of unfertilized and aged unfertilized eggs of* Paracentrotus lividus.

Total homogenate		Particulate		TCA-soluble	
Unfert.	Aged unfert.	Unfert.	Aged unfert.	Unfert.	Aged unfert.
127	137	19.2	37.2	432	312
98.9	86	39	60	327	215
611	549	105	109.4	1902	1347

TABLE II. *Specific activity (counts/min/1 mg N) of the particulate and TCA-soluble fractions of eggs of* Paracentrotus lividus *unfertilized and 15 minutes after fertilization.*

Particulate		TCA-soluble	
Unfertilized	Fertilized	Unfertilized	Fertilized
—	—	243	323
—	—	702	820
95	127	1038	1180
73	135	1100	1340

increase of the specific activity of the particulate fraction and of the TCA-soluble fraction takes place.

Changes during early development.—After some preliminary experiments in which only a few developmental stages were examined, three experiments were carried out in which the activity of the different cell components was followed at close and constant time intervals up to 18 hours. The following results have been obtained.

The activity of the particulate fraction which, as already mentioned, is very low in the unfertilized egg, increases exponentially during the first six hours of development, i.e. until the early blastula stage. From this point on, the activity increases almost linearly. On the whole, an S-shaped curve is obtained (Fig. 1).

As described previously, the TCA-soluble fraction contains, in the unfertilized egg, by far the largest proportion of the radioactivity of the egg. However, after the temporary increase observed in this fraction immediately

after fertilization, its activity, as indicated in Table III and in Fig. 2, begins to decrease, at first quite rapidly and, as development proceeds, more slowly.

The supernatant obtained after the removal of the particulate sedimenting at 24,000 *g* ("supernatant second") contains, apart from the TCA-soluble

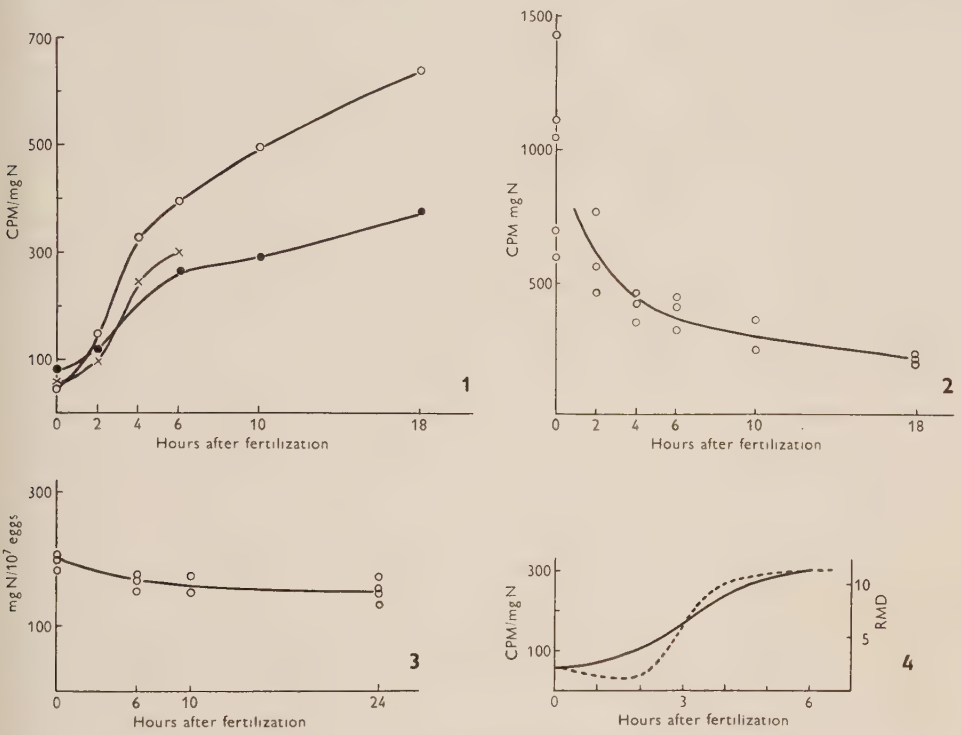

Fig. 1.—Uptake of the isotope by the particulate fraction of developmental stages of *Paracentrotus lividus*. Each curve refers to a separate experiment.

Fig. 2.—Decrease of the isotope content of the TCA-soluble fraction during development of *Paracentrotus lividus*.

Fig. 3.—Variation of the total nitrogen content per embryo during development of *Paracentrotus lividus*.

Fig. 4.—Comparison of the curves of the increase of the relative mitochondrial density, RMD (dotted line, redrawn from Gustafson and Lenicque [6]), and of the uptake of the isotope (solid line) by the particulate fraction during the first six hours of development of *Paracentrotus lividus*.

material, soluble proteins and microsomes. For technical reasons the separation of these two components has not yet been done. In some preliminary experiments this fraction was submitted to dialysis and the activity of the non-diffusible part measured. This appears to increase parallel to the activity

TABLE III. *Isotope content of the TCA-soluble fraction during development of* Paracentrotus lividus, *as per cent of the activity of the total homogenate.*

The values on the same horizontal line belong to the same experiment. The data are the experimental values, i.e. counts/min/0.1 ml (corrected for background) of the total homogenates and of the TCA-soluble fraction (this latter corrected also for dilution) prepared as described in the text.

Unfertilized		2 hours		4 hours		6 hours		12 hours		18 hours	
Tot. hom.	TCA-sol.	Tot. hom.	TCA-sol.	Tot. hom.	TCA-sol.	Tot. hom.	TCA-sol.	Tot. hom.	TCA-sol.	Tot. hom.	TCA-sol.
59	44 (74.5)[a]	62	30 (48.4)	51	18 (35.3)	68	18 (26.4)	67	10.5 (15.7)	75	10.5 (14.0)
128	106 (82.6)	174	98 (56.2)	146	54 (37.0)	166	39 (23.4)	125	26.5 (21.2)	187	19.3 (10.3)
71	60 (84.5)	65	45 (69.2)	68	36 (53.0)	71	33 (46.4)				
122	95 (78.5)					130	37 (28.5)				
98	90 (91.8)					96	35 (36.5)			72	16 (22.2)
74	68 (91.8)							78	7 (9.0)		

[a] Figures in parentheses indicate percentages.

of the particulate fraction and a maximum is reached at about 6 hours of development. This question, however, will be submitted to further, more detailed analysis.

Control Experiments

Contamination versus incorporation.—In order to rule out the possibility that our results might be altered by the contamination from some isotope present in the jelly coat, incorporated or simply adsorbed, this has been removed by acid treatment, concentrated by ethanol precipitation and its radioactivity determined. As previously reported [15], no activity has been found in any of such preparations. Furthermore, in one experiment two animals were injected with 5 μC of S^{35}-methionine each and were open immediately afterwards, the gonads removed and the eggs collected in the usual way for the control of radioactivity. No appreciable radioactivity was found in this preparation.

Nitrogen content per embryo during development.—As in our experiments

total nitrogen was taken as a reference, it seemed important to ascertain whether this reference point was subject to any change in the course of development, especially in view of the conflicting statements of a constancy of this value [4] and, on the other hand, of a loss of ammonia in the course of development [10].

Therefore four experiments were carried out in which the total nitrogen per egg was determined. Each time four samples were collected from the same batch of eggs, i.e. one before fertilization (after removal of the jelly coat), and the other three in the course of development up to 24 hours. As it is shown in Fig. 3, a slight decrease of total nitrogen per embryo has been found to take place in the course of development. At the same time, in all our experiments, a slight but constant increase of the calculated specific activity of the total homogenate has been detected. However, the agreement between the two groups of data is not entirely satisfactory, as the increase in specific activity has always been somewhat higher than would be expected on the basis of the decrease of total nitrogen. This point is left open for further investigations.

DISCUSSION

The first point of our results to which we would like to draw attention is that in the unfertilized egg by far the largest uptake of radioisotope is by the TCA-soluble fraction, whereas other cell components have a very slight uptake. This situation does not undergo substantial changes upon aging of labelled unfertilized eggs. However, as soon as the egg is activated, a lively turnover is started which is especially relevant in the particulate fraction, apparently at the expense of the TCA-soluble fraction.

A few remarks seem to be necessary here about the composition of the particulate fraction. In the present experiments no special care has been taken for the purification of this fraction which, however, reveals no contamination with yolk granules, and observations with the electron microscope show that it results largely from mitochondria. It must be emphasized that no data are at present available as to the composition of this fraction during development. In fact the possibility should be considered that it may change during development. If this were the case, a correction might be necessary to our calculations of specific activity.

Comparison of our results with those of previous investigators is rendered difficult by the differences in the technique, which have already been outlined. Some points, however, need to be discussed.

Hultin [8] giving N^{15}-labelled ammonia to unfertilized and developing eggs of *Paracentrotus lividus*, apart from a generally more active incorporation in developing than in unfertilized eggs, found that the greatest incorporation of the isotope takes place in the TCA-soluble fraction. This incorporation reaches a maximum in the 10-hour embryos and then falls as development proceeds. The incorporation in the soluble proteins begins to be especially active after about 25 hours of development, a period which is beyond the stages we have considered in the present experiments. Also Hoberman *et al.* [7] found that the incorporation of deuterium in the proteins of *Arbacia* embryos becomes markedly accelerated at the blastula stage. In other experiments in which N^{15}-alanine was given to eggs and embryos [9] during early development, a rapid uptake was first found to occur in the fraction containing submicroscopic granules. At the mesenchyme blastula stage a considerable uptake by the mitochondria was observed. This last result had been considered to be due to the increase of the mitochondrial population which Gustafson and Lenicque [5, 6] found to occur at this stage. The observations of these authors indicate in fact that the relative density of the mitochondrial population, which remains almost constant during the first 2 to 3 hours, shows next a sharp increase followed by a plateau from the 4th to the 10th hour (i.e. to the mesenchyme blastula stage), after which a new and considerable increase takes place. The increase of the mitochondrial population during cleavage, however, has not been observed by Shaver [18] in *Lytechinus* and *Strongylocentrotus*.

Our results show that the isotope uptake of the mitochondria, which is negligible in the unfertilized egg, is activated during cleavage stages, and in fact immediately after fertilization. However, if our data are plotted on the same scale, together with those of Gustafson and Lenicque [6], a remarkable agreement between the two curves is evident (Fig. 4). The only point at variance concerns the first two hours of development during which the former authors did not find any increase in the mitochondrial population.

The investigations of Gustafson and Hasselberg [4], carried out on total homogenates of developmental stages of sea urchin, would suggest a fairly late activation of the mitochondrial enzymes which should take place as late as the beginning of the mesenchyme blastula. On the other hand, experiments carried out in this laboratory on isolated mitochondria [12, 13, 14] have indicated that changes are taking place in some mitochondrial enzymes (one protease and adenosinetriphosphatase) immediately after fertilization. Permeability tests indicate, furthermore, that at the same time changes occur in the properties of the mitochondrial membrane [13, 14]. Our findings

that the activity of the mitochondria, as indicated by their isotope uptake, sets in immediately upon fertilization and is then continuously accelerated are in line with the above results and may lead one to suggest an activation of the mitochondria upon fertilization. It seems interesting to add here that experiments in progress by one of us (E. N.) indicate a parallel increase of the apparent –SH groups of the mitochondria.

It seems also appropriate to draw attention to the similarity of the S-shaped curve of the increase of mitochondrial activity during development to the curve of respiration of the developing sea urchin egg [11, 1].

Our observations have also shown that in the unfertilized egg the isotope is almost entirely stored in the TCA-soluble fraction which thus represents a sort of metabolic pool from which the embryo draws during its development, especially during the early stages. The similarity with what happens in micro-organisms during protein synthesis (see [2, 19, 3]) cannot escape attention. The temporary increase of the activity of this fraction immediately after fertilization may be correlated with the increase in total- and amino-nitrogen which has been shown to occur in this fraction immediately after fertilization [16, 17]. Soon afterwards, however, an inverse process is started, namely the decrease of the activity of this fraction while an increase is shown in other fractions. This result may be suggestive of synthetic processes starting early in development.

SUMMARY

S^{35}-labelled methionine has been incorporated in the unfertilized eggs of *Paracentrotus lividus* and the distribution of the isotope among cell components has been studied during early development.

In the unfertilized egg the isotope is largely stored in the fraction soluble in 10 per cent TCA (i.e. the so called non-protein fraction). Initiation of development is characterized by a depletion of this fraction which is very rapid during cleavage stages. At the same time a lively uptake of the isotope takes place in the mitochondria. The possible significance of these results is discussed.

The technical assistance of Mr. O. A. Oliva is acknowledged.

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CINÉ-MICROGRAPHIC STUDIES ON MITOSIS IN ENDOSPERM

IV. THE MITOTIC CONTRACTION STAGE[1]

A. BAJER

Laboratory of Plant Physiology, Jagellonian University, Cracow, Poland

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In the preceding paper of this series [2] consideration was given to the clear zone, a region of the cell which was shown to be the cytoplasmic constituent of the spindle. In the present paper the same technique of time-lapse photography with subsequent ciné-projection and frame-by-frame analysis was used, but attention was directed to the dissolution of the nuclear membrane and the subsequent contraction stage.

OBSERVATIONS

Chromosomal movements before the contraction stage.—During prophase the change in shape of the chromosomes leads to some slight alteration in their arrangement, but movements of this kind were not analysed. Analysis of the record was begun at a later stage, when the chromosomes are easily distinguished and are shortening more quickly. In these later stages of prophase no chromosomal movements were observed in the projected film.

Some exceptions did occur in several nuclei of *Clivia*, which were not otherwise abnormal but proceeded regularly through the remainder of mitosis. In these exceptional prophases the chromosomes move conspicuously, oscillating irregularly without shifting far or causing the general arrangement of the group to change. It is probable that the oscillating chromosomes rotated on their long axes, but this could not be ascertained.

The causes of these movements are obscure but there are three possible explanations. (1) The chromosomal arms may be in Brownian movement; (2) the arms may be displaced by currents in the nuclear sap at a time when its physical state is changing; (3) the internal structure of the chromosomes may be altering e.g. spiralisation. Since not all prophase nuclei in *Clivia* show these movements and since they do not appear in any cells in some species with smaller chromosomes, the first explanation does not seem probable. The second also is unlikely because the fine intranuclear particles, which are in Brownian movement, do not show the influence of currents. Thus the third hypothesis is preferred.

[1] This paper, as well as Nos. III and V of the series, are concerned mainly with the analysis of the film *Mitosis in Endosperm*, made by A. Bajer and J. Molé-Bajer (Film Polski, 1955). Sequences of this film are here referred to by the numbers printed in the film itself. The author is especially grateful to Dr. J. Boss and Dr. H. B. Fell from Strangeways Research Laboratory, Cambridge, England, for most valuable suggestions and much help during the preparation of the manuscripts of this series (III–V).

The contraction stage of mitosis.—The contraction stage has already been studied in detail [1, 6] and its causation has been discussed. The contraction stage was defined as "the stage lasting a few minutes prior to prometaphase (metakinesis), when the scattered chromosomes which had in late prophase formed a loose ball aggregate suddenly in a tighter central mass" ([6], p. 561).

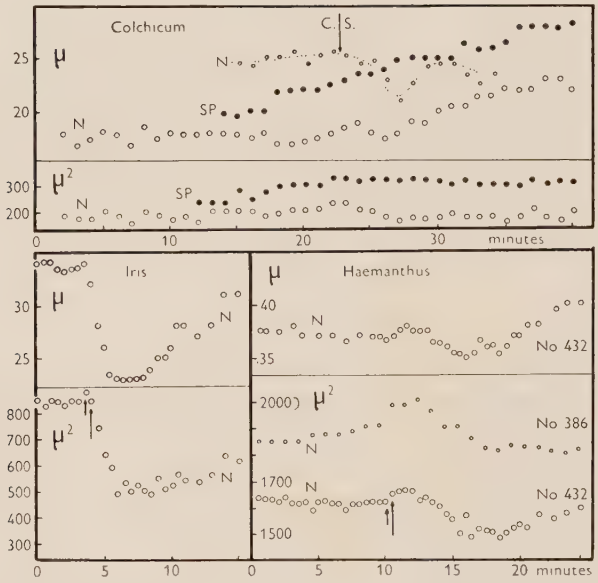

Fig. 1.—*Concentraction stage and spindle formation.* Diameter in μ measured parallel to the future long axis of the spindle, and cross-sectional area in μ^2, of the clear space and, later, the spindle. *SP*, clear space and spindle. *N*, nucleus, and then the chromosome group (hollow circles). *Colchicum. CS*, contraction stage. Large hollow circles: cell No. 36 (No. 17 in the film). Small hollow circles: cell No. 67a (No. 18 in film). Aggregation is strong in cell 67a but weak in No. 36. *Iris* (cell 94, No. 14 in film) and *Haemanthus* (cells 423 and 386, Nos. 25 and 36 respectively in film). Short arrow: nuclear membrane still present. Long arrow: nuclear membrane already disappeared. An increase in nuclear area just after the nuclear membrane disintegrates is indicated in the graph for *Haemanthus* but not in that for *Iris*, although in the latter the increase was plainly seen on projection. (Exceptionally, this increase is detectable in the graph of change in nuclear diameter as well as in that of area.)

In the light of present observations this definition should now be amplified (cf. p. 247). It was also suggested that after the contraction stage the "interaction between the spindle and the chromosomes begins" and that immediately after the contraction stage "the guiding role of kinetochores in chromosome movements during mitosis first manifests" itself ([1], p. 403), though the exact time of this process was not determined.

In their earlier work the course of the contraction stage was not investigated

in cells with small chromosomes. Observations on *Colchicum* and *Daphne*, which have small chromosomes, indicate that the contraction stage occurs in these plants also and most nearly resembles that in *Iris* and *Clivia* (Fig. 1), among the plants with larger chromosomes. The chromosomal movements of prometaphase also follow a similar course, although observations on the individual arms [3] of small chromosomes cannot be made. Thus it can be seen that the contraction stage occurs in nuclei with different structure.

The disruption of the nuclear membrane.—The nuclear membrane breaks down when the clear zone has reached its maximum size. The disappearance of the membrane is especially clearly seen in cells which have large chromosomes and a well developed clear space. The cells of *Haemanthus* proved to be the most suitable for observing this change, and the exact time at which the membrane was lost could be estimated within $\frac{1}{2}$–2 minutes. The nuclear membrane fades gradually, the change not always proceeding uniformly around the nucleus. As the membrane disappears, the volume of the nucleus increases giving the impression of a temporary enlargement (Fig. 1, *Haemanthus*); this is seen best in those cells in which the change in area of the nucleus may be considered as a measure of change in volume i.e. in very flattened cells (cf. [2], Fig. 2), and less well in other cells. The change in volume lasts a few minutes; it is probable that the increase in the nuclear volume begins 1–2 minutes before the final disappearance of the membrane. The chromosome group usually reaches its maximum size after the loss of the nuclear membrane, and then diminishes in diameter with the onset of aggregation (cf. definition of contraction stage, p. 246). The aggregation depends on many factors previously analysed in detail [6] and is least in flattened cells where the decrease may not exceed about 10 per cent of the whole nuclear volume. The preceding increase in nuclear territory is usually not large, about 4–8 per cent in *Haemanthus*. The course of the contraction stage depends mainly on mechanical conditions in the cell. It was also found that the direction in which the chromosomes move during aggregation partly determines the direction of the long axis of the spindle. The course of the contraction stage probably influences also the duration of prometaphase which is longer after a weak contraction than after a strong one. The problems of the control of the duration of metaphase and prometaphase, and of the orientation of the spindle, however, are far from being elucidated.

It is obvious that enlargement of the nuclear territory, being proportionately small, is more easily observed in large cells such as those of *Haemanthus*, after flattening. In flattened cells the contraction stage is usually less marked, although there is no difficulty in noting its duration. In cells with small and

medium sized nuclei (*Colchicum, Iris, Clivia*) the increase in area occupied by nuclear material was so small as to lie within the limits of the error of measurement, although it could be seen during projection.

The small proportion by which the nucleus increases in volume explains why the increase is only in exceptional cases indicated in linear dimensions (Fig. 1, cell No. 432), and why it was not reported in previous papers [1, 6]. For measurement and exact observations *Haemanthus* again proved best. It was found that in this species the increase of the area occupied by the chromosomes is the result of a temporary straightening of chromosomal arms at the periphery of the nucleus, where the nuclear membrane has recently existed. It appears that after the mechanical barrier of the nuclear membrane is broken the chromosomes may straighten partly as the result of their own resilience and stiffness; the chromosomes at the centre of the group do not move at all.

Random movements during the contraction stage.—During time-lapse cinémicrography only a few micrographs (1.5–12) are made per minute, and therefore during projection the results of Brownian movement, but not the movements themselves, are observed; these are called "random movements". Furthermore, errors arise in measuring true Brownian movement on the film, because of the difficulty of placing successive frames sufficiently exactly in the same position.

In previous papers [1, 6] it was suggested that during the contraction stage changes occur in the fine structure of the nucleoplasm and clear space, leading to the development of a definite gelated and submicroscopically oriented structure. The present observations provide further support for this view.

In not more than one fifth of all *Clivia* cells in prophase the nuclei contain tiny particles in Brownian movement. Usually there are 1–3 of these particles and never more than five; they indicate no abnormality, and cells containing them pass normally through mitosis.

In *Haemanthus* also such particles were found but unfortunately they changed position so much that they could not be observed for more than short periods. The particles were usually seen at all stages of mitosis in *Leucojum* which is very useful for observing Brownian movement in anaphase, but the large size of the chromosomes in relation to the volume of

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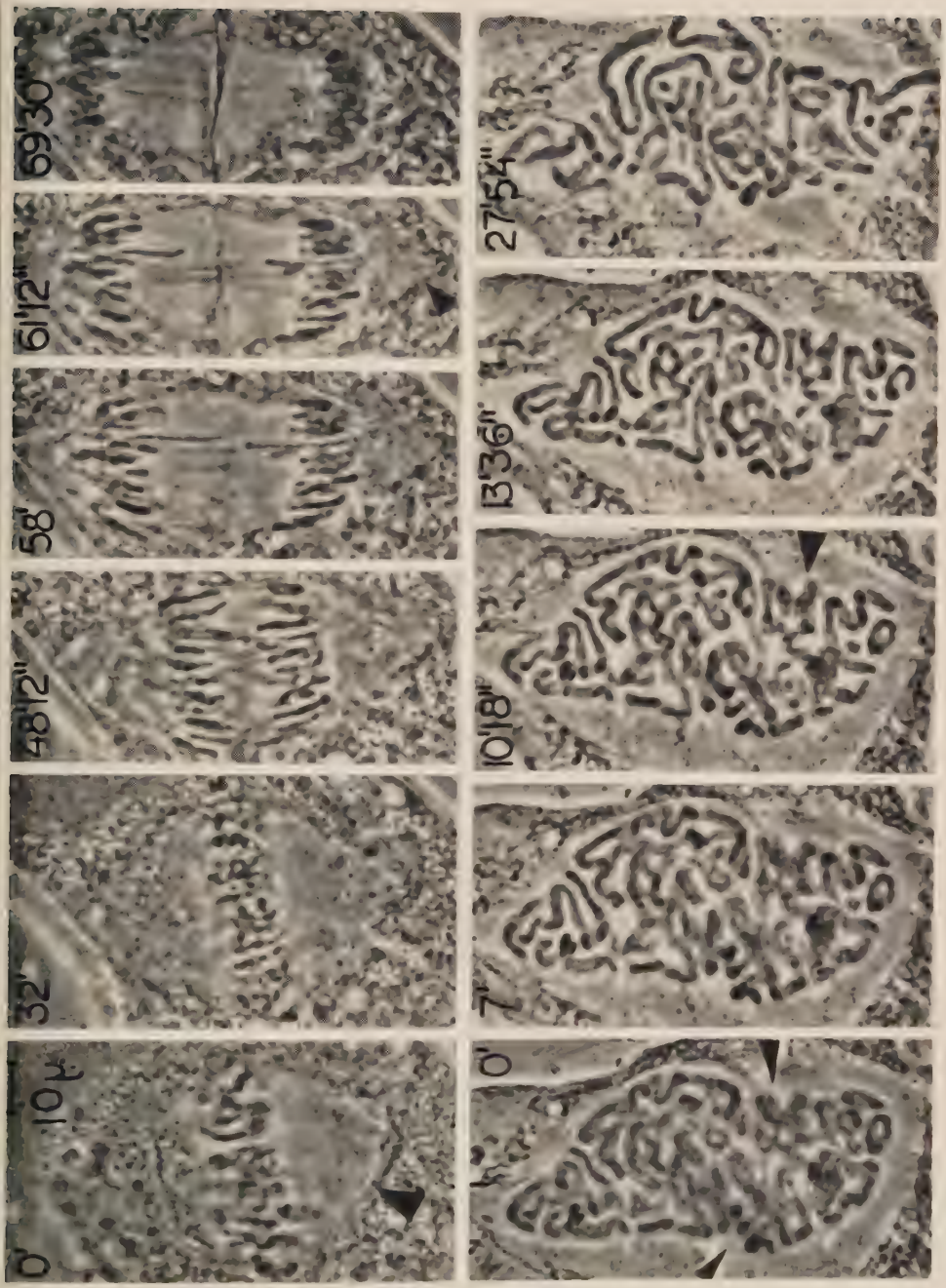
Fig. 2.—Rupture of nuclear membrane in *Haemanthus* (cell 386, No. 36 in film): lower row. Upper row: the sharp border between the half-spindle and cytoplasm during anaphase in *Clivia* (cell 112, No. 11 in film). Living cells. Phase contrast. Timing indicated on micrographs.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the nucleus and, later, to that of the spindle leaves little room for free movement in either prophase or prometaphase. For the study of Brownian movement *Clivia* was best, and *Iris* and *Colchicum* least suitable.

During prophase the particles in *Clivia* oscillate in all directions, their mean velocity being 7–8 μ/min. Most particles move irregularly, and wide

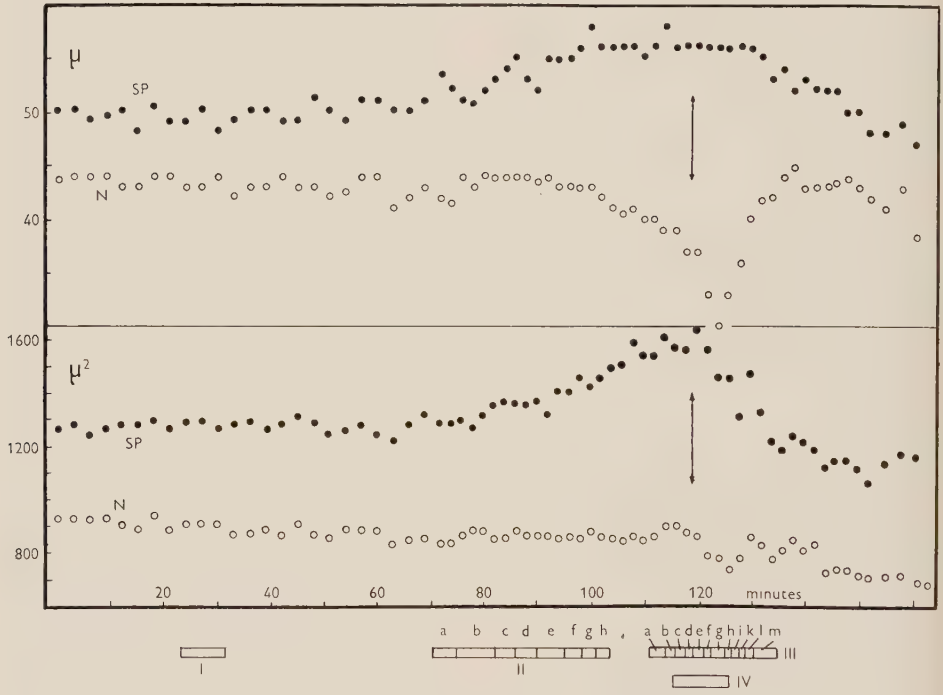


Fig. 3.—*Spindle formation and contraction stage in Clivia.* Cell 192: No. 11 in film. For key see legend to Fig. 1. The arrows indicate the time of loss of the nuclear membrane. The nuclear diameter decreases somewhat before the contraction stage, owing to rounding of the nucleus. The small rectangles I IV indicate the periods of observation of four oscillating particles, the paths of which are the subject of Figs. 4 and 5.

oscillations may alternate either with smaller ones or with complete rest. During projection one does not have the impression that the range or frequency of the oscillations diminishes in late prophase before the contraction stage, but graphs derived from the film (Fig. 5) may perhaps indicate some such slight diminution. Unfortunately particles in random movement are very rare and it is difficult to say whether the example on which Figs. 3–5 are based is normal or exceptional; some slow change of position seems probable. In the contraction stage, however, the behaviour of the particles

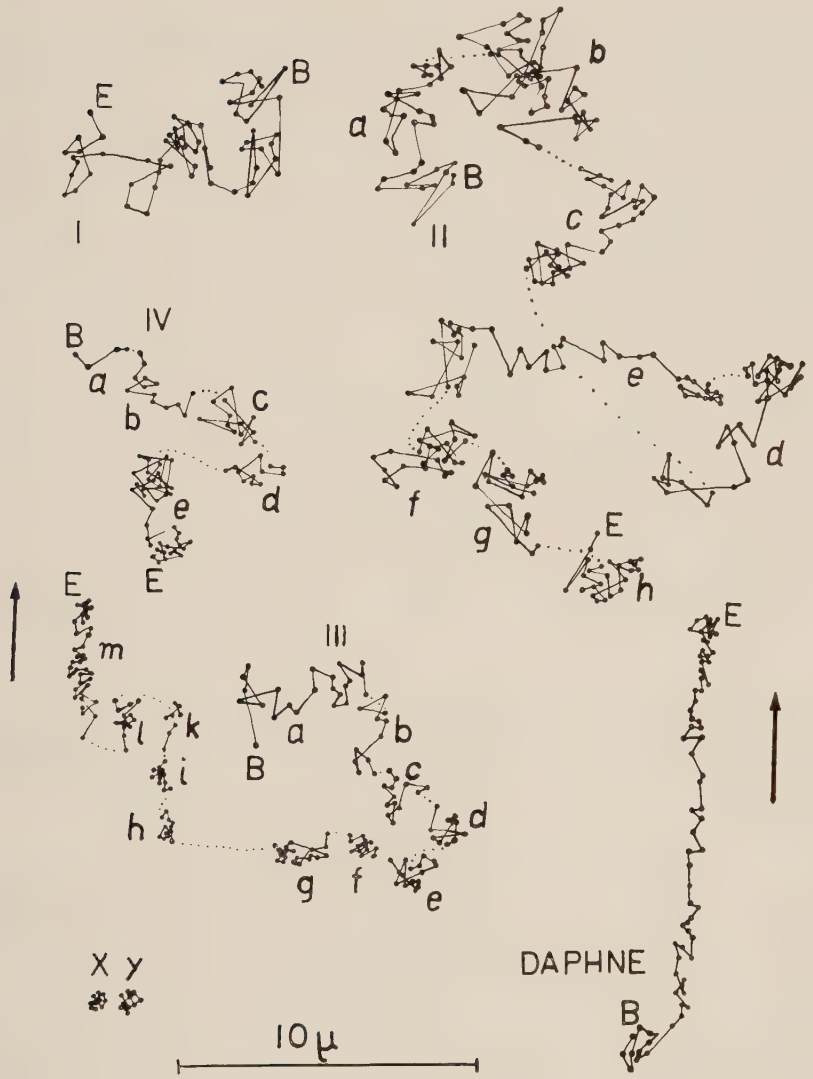


Fig. 4.—*The path of oscillating particles in prophase, contraction stage and prometaphase.* The arrows indicate the direction of long axis of the spindle, and are directed towards the pole. The dots on the paths represent $\frac{1}{8}$ minute intervals. (B beginning. E = end of measurement.) *Clivia.* Cell 192. Times of observation indicated by the four small rectangles at the bottom of Fig. 3 (I–IV). Lower right-hand corner: *Daphne mezereum;* late prometaphase. (Cell 39 of film.) *X–Y* indicates the error in plotting. *X* and *Y* are two particles whose position was recorded several times after removal and replacement of the paper, the same frame of the film being used.

alters abruptly, for they then stop oscillating and may even remain quite motionless for some minutes; such slight movements as take place are parallel with the long axis of the spindle. It is characteristic of the movements of the particles in prometaphase that they are usually in the direction of the spindle poles. The diminution in the movement of the particles is so striking

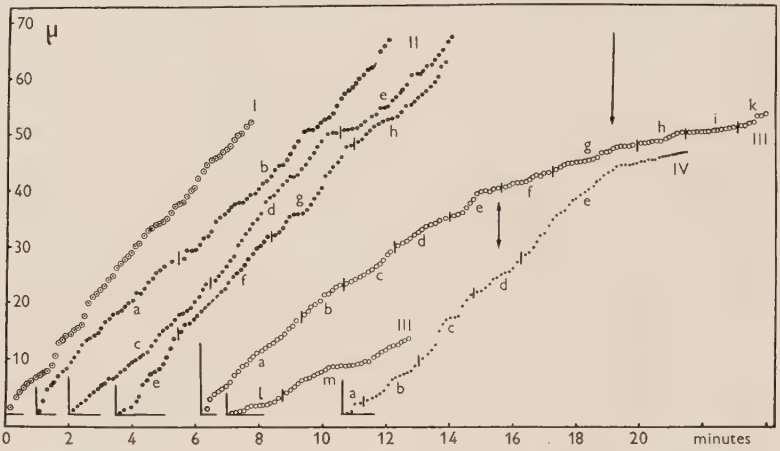


Fig. 5.—*Oscillation of particles in prophase, contraction stage and prometaphase* ("random movement"). Distances moved by particles, the paths of which are given in Fig. 4, plotted cumulatively against time. The slope of the curves in prophase (I and II) is plainly different from the slope in the contraction stage and prometaphase (III and IV). Short arrow: time of greatest aggregation of chromosome group. Long arrow: time after which further oscillation of the particles appears to cease in the projected film. The acceleration of movement in III corresponds to a movement towards the pole.

that the projected film gives the impression of a sudden arrest of motion as the contraction stage begins. The change from the oscillations of prophase can be seen both in the plot of the path of a particle, and in a graph of the distance over which the particle has moved (Fig. 5).

While the measurement of the path of a particle involves an appreciable error, this is constant throughout and cannot affect the difference between prophase and prometaphase as seen in either the plots of the paths or in the graphs, even though the error affects the numerical results.

The stretching of kinetochores.—In early prometaphase, immediately after the contraction stage or a little later, the kinetochores were seen to behave very curiously in some cells. They expanded abruptly and markedly across the long axis of the chromosomes and approximately along the long axis of the spindle, but not always simultaneously in all chromosomes. In this way each kinetochore appeared to develop a hole which attained its

greatest diameter about five minutes after the beginning of stretching (Fig. 6) and then gradually diminished until it was seen as a light spot at the time when the chromosomes were moving most quickly. Often, even the light spot disappeared. In *Haemanthus* stretching usually lasted about ten minutes (Fig. 6). The process could not always be clearly followed, and it is not known whether it occurred in all the cells examined; until now it has been seen only in *Haemanthus*, which has the largest chromosomes of all the plants studied. The question arises as to what forces are responsible for this stretching. The forces causing chromosomal movements can stretch the limbs of chromosomes, as is shown by bridges in anaphase. In prometaphase it is only after the stretching of the kinetochores that the latter lead chromosome movements; this suggests that the stretching of the kinetochores may mark the beginning of their interaction with the spindle.

These observations resemble some of Lima-de-Faria's descriptions [9], and support some of his conclusions, especially that the kinetochore is divided before metaphase and kept together by the neighbouring parts of the limbs. The present observations are similar also to those of Davies [7], but unlike his observations, ours were made on normal cells.

DISCUSSION

It has been shown that the nuclear membrane breaks down just before the contraction stage. It remains to consider whether there is a membrane around the spindle. Wada [10, 11] believes that such a membrane exists as a phase boundary, and maintains this view in his most recent paper [12]. On the basis of observations on the fern *Hymenophyllum* [4, 5] we have supported Wada's opinion. On the other hand, it is proved beyond doubt that in endosperm the nuclear membrane disappears at the time of its rupture, no exception to this having been seen. Previously [1] the moment of breaking was supposed to be at some time before the contraction stage, but it seems probable that in some endosperm cells a membrane of the nature of a phase boundary probably envelopes the mitotic spindle (half spindles). In some cells in anaphase it was possible to trace very exactly the piercing of the cytoplasm by the half-spindles, the border of the latter forming a sharp boundary (membrane?) between the spindle and outer cytoplasm. The border between the spindle and outer cytoplasm may also be sharp and well defined in prometaphase and metaphase. Such a demarcation is, nevertheless, very rare, and in most cells there is no continuous transition between cytoplasm and spindle. It may be stated in conclusion that the nuclear membrane dis-

appears just before the contraction stage, although, under particular cytoplasmic conditions which cannot at present be more exactly defined, there may appear a secondary membrane having the character of a phase boundary. This is different in origin from the nuclear membrane and not a later form of existence of it.

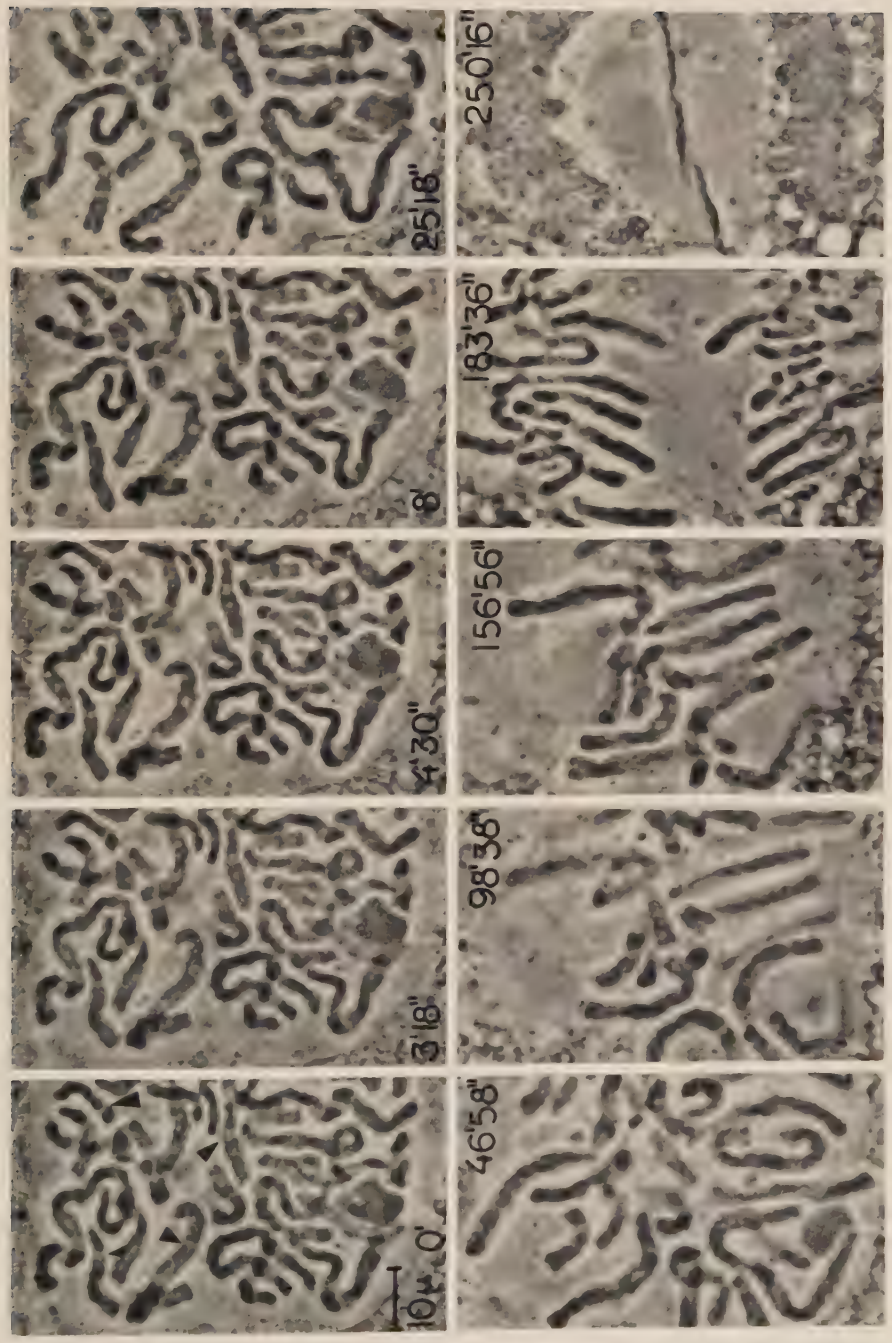
After the contraction stage the further differentiation of the spindle, which is probably assuming increasingly the submicroscopic structure indicated by Inoué's studies with the polarizing microscope is likely to be associated with kinetochore activity. The differentiation does not proceed equally in all parts of the spindle but is most advanced near kinetochores and remains so. This in turn is related to the determination of the long axis of the spindle and of the time at which this is first manifested. Observations on the random movement of small particles indicate that the spindle begins to assume its characteristic structure during the contraction stage, but no information was obtained as to how the direction of its long axis is determined. This probably depends on the shape of the cell, the development of the clear zone and the arrangement of kinetochores within the prophase nucleus. The following observation suggests that even in prometaphase the orientation of the long axis of the spindle is not completely established: if a cell contains more than one spindle and these spindles, whether parallel or not, fuse, then the spindle so formed is oriented in a direction different from the direction of the compound spindles before fusion.

SUMMARY

1. The nuclear membrane breaks down when the clear zone reaches its maximal size. Just after the membrane disintegrates and immediately before the contraction stage, the nuclear sap begins to mix with the substance of the clear space. The rupture of the nuclear membrane initiates the contraction stage.

2. After the loss of the mechanical barrier provided by the nuclear membrane, chromosomal limbs at the periphery of the group straighten slightly, so that the chromosomes come to occupy a slightly greater apparent area. Then follows a reduction in the space occupied by the chromosomes: the contraction stage. This apparent compression of the group is followed by

Fig. 6.—*Stretching of kinetochores after the contraction stage.* The diameter of the kinetochores increases and then diminishes. Kinetochores indicated by arrows. The time is indicated on the micrographs; at the 159th minute anaphase begins. *Haemanthus*, cell 410, No. 41 in film. Phase contrast.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simultaneous straightening of chromosome limbs and stretching of kinetochores. After this stretching the kinetochores begin to lead the movements of the chromosomes; it is suggested that the kinetochores are stretched when they first become attached to the spindle. During contraction stage the spindle gelifies, after which an orientated structure is manifested.

3. The extent of the irregular movements of small particles decreases after the contraction stage; when the particles begin to oscillate parallel to the long axis of the spindle.

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Experimental Cell Research **14**, 257–267 (1958)

EFFECTS OF CYANIDE AND IONISING RADIATION ON THE ROOTS OF *VICIA FABA*[1]

LORNA J. LILLY[2]

Department of Genetics, The University, Sheffield, England

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In 1935 Mottram [13] demonstrated that cyanide treatment increased the inhibition of growth caused by the irradiation of *Vicia* roots. Thoday (unpublished) endeavoured to show that cyanide also increased the numbers of chromosome aberrations caused by irradiation, but was unable to demonstrate this. There was therefore an apparent conflict, for Thoday [16] had shown that there was a high correlation between chromosome aberrations and growth rate reaction of *Vicia* roots. The present work was therefore undertaken to test Mottram's work on a larger scale, and to investigate the cytological effects of combined cyanide and radiation treatments.

EXPERIMENTAL METHODS

Culture of the beans.—Suttons Prolific Long Pod beans were used throughout the experiment. They were soaked, peeled and germinated in sand as suggested by Read [14]. They were then transferred to an aerated and thermostatically controlled culture tank, where they were grown in the dark.

Irradiation with X-rays.—The X-rays were given at the Sheffield Radium Centre, with a 220 K.V. Peak Newton Victor X-ray apparatus, with a half value layer of 1.5 mm Cu. A perspex cell was built to hold the beans during radiation. It was constructed so that the root tips were held in the centre of the radiation field, and was divided into two compartments, one containing the cyanide solution (pH 7) and the other water. The cotyledons of the beans rested along the top of the cell, while the roots hung down into the cyanide or water solutions. A stream of oxygen or nitrogen was bubbled through these solutions. The beans were exposed to the appropriate gas and solution for a total of thirty minutes, fifteen of which were prior to irradiation.

Irradiation with α-particles.—The α-particle irradiation was carried out by Dr. L. H. Gray at Hammersmith Hospital. The technique used was similar to that described by Gray and Scholes [6].

Cytological techniques.—Root tips were fixed in acetic alcohol, stained by the Feulgen technique of Darlington and LaCour [5], and made into root tip squashes.

[1] This work forms part of a thesis accepted for the degree of Ph.D. of Sheffield University.
[2] Present address: Department of Genetics, The University, Glasgow.

Fixation times were 1½, 3, 6, 9, 12, 16, 24, 36 and 48 hours after oxygen treatment, and 3, 4½, 6, 7½, 9, 10½, 12, 16 and 24 hours after nitrogen treatments. One hundred anaphases from each root were examined for abnormalities (except in roots lacking this number). Estimations of the mitotic indices (the proportion of cells in metaphase, anaphase and telophase) were also made.

EXPERIMENTAL RESULTS

The consequences of treatment with cyanide in the absence of radiation and the influence of cyanide upon radiosensitivity were both investigated by measuring inhibition of growth, increase in the number of abnormal anaphases present, and inhibition of mitosis.

The action of cyanide in the absence of radiation

The experiments involve four conditions. Roots were immersed in water or in 5×10^{-4} *M* potassium cyanide at pH 7 for 30 minutes and, at the same time, nitrogen or oxygen was bubbled through the solutions.

The growth rate experiments.—Figs. 1 and 2 show curves representing the growth of 15 beans in each of the four experimental conditions. It seems clear that, in the presence of oxygen, cyanide limits the growth of beans, and a polynomial analysis of the data reveals that the linear and cubic components of the oxygen plus cyanide curve differ significantly from those of the other three curves ($P = 0.001$). There is some suggestion that the combination of cyanide with nitrogen may also cause a slight reduction in growth, but this effect is certainly much less striking than the effects of cyanide in the presence of oxygen.

The cytological experiments.—An account of the cytological action of cyanide has been published elsewhere [9]. Briefly, there is a radiomimetic action of cyanide in the presence of oxygen. This action of cyanide was detected as an increase in the number of abnormal anaphases. Supporting evidence for this observation is found in a parallel increase in micronuclei. There is also some disturbance of nucleic acid metabolism giving stickiness and spurious bridges, in slides fixed at 1½, 3 and 6 hours after treatment with oxygen and cyanide. At later fixation times the abnormalities are mainly fragments. Kihlman [7], who has recently confirmed these cytological observations, has also succeeded in establishing that there is a very small but significant action of cyanide in the presence of nitrogen.

Mitotic index counts indicate that there is a marked inhibition of mitosis at 6, 9 and 12 hours after cyanide plus oxygen treatment, which is not found after water plus oxygen treatment. After both the treatments involving nitro-

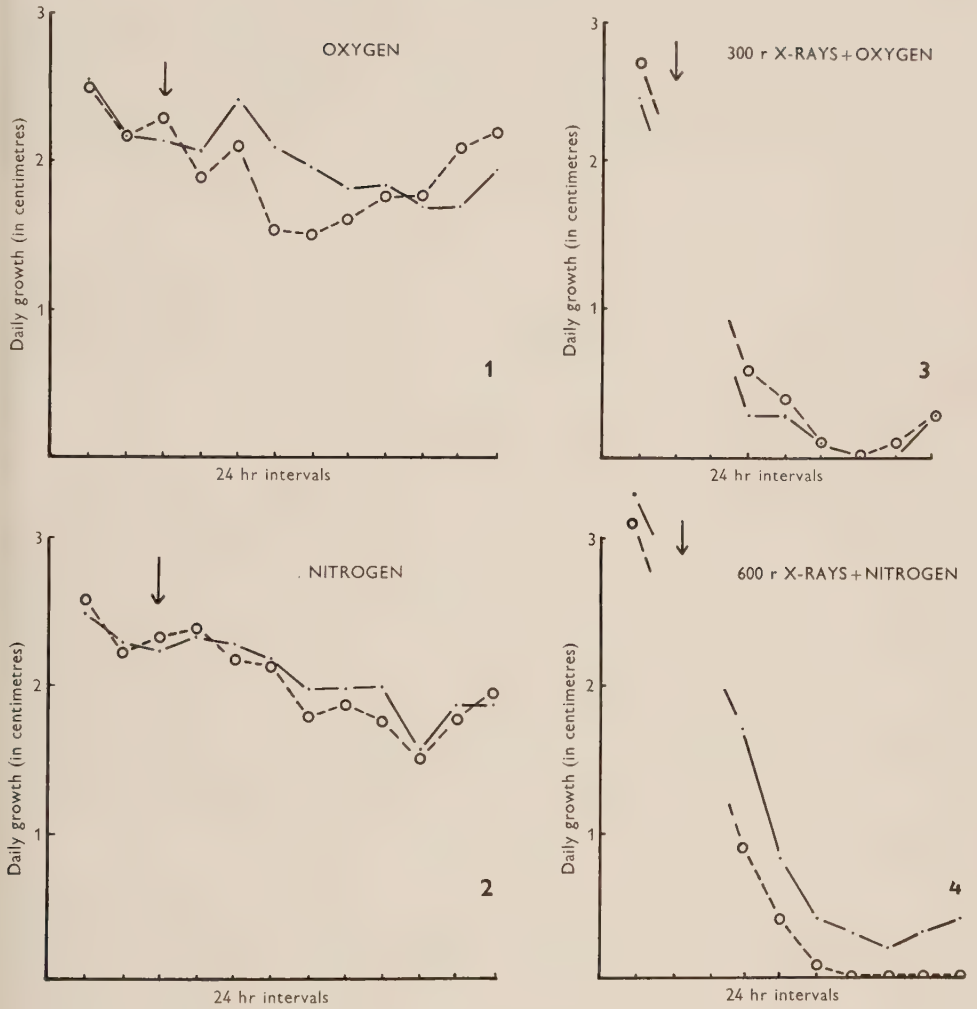

Fig. 1.—Daily growth increments of beans treated with 5×10^{-4} potassium cyanide (○ – – – – ○), or water (· ——— ·), in the presence of oxygen. The arrow indicates the time of treatment.

Fig. 2.—Daily growth increments of beans treated with 5×10^{-4} potassium cyanide (○ – – – – ○), or water (· ——— ·), in the presence of nitrogen. The arrow indicates the time of treatment.

Fig. 3.—Daily growth increments of beans irradiated with 300 r X-rays (42.8 r/min) during treatment with 5×10^{-4} potassium cyanide (○ – – – – ○), or water (· ——— ·), in the presence of oxygen. The arrow indicates the time of treatment. The beans were not measured on the day of treatment.

Fig. 4.—Daily growth increments of beans irradiated with 600 r X-rays (43.5 r/min) during treatment with 5×10^{-4} potassium cyanide (○ – – – – ○), or water (· ——— ·), in the presence of nitrogen. The arrow indicates the time of treatment. The beans were not measured on the day of treatment.

gen, there is an initial inhibition of mitosis followed by recovery at 10 hours. The mitotic index after cyanide plus nitrogen treatment is only slightly lower than that following water plus nitrogen treatment.

There is, then, a radiomimetic action of cyanide which is oxygen sensitive, and which is reflected in inhibition of growth and mitosis and in an increase in the proportion of anaphases that have bridges and fragments.

The action of cyanide in the presence of X-rays

As before, beans were immersed in cyanide or water solutions for 30 minutes and at the same time oxygen or nitrogen were bubbled through these solutions. Fifteen minutes after the commencement of these treatments the exposure to radiation began. The X-ray doses and the concentrations of the cyanide solutions used, are given below.

The growth rate experiments.—Fig. 3 shows the daily growth increments of beans X-rayed in water or cyanide solutions through which *oxygen* was *bubbled.* ($KCN = 5 \times 10^{-4}$ *M*; 300 r X-rays at 42.8 r/min). There is no evidence that cyanide increases the inhibition of growth caused by X-rays.

Fig. 4 shows the daily growth increments of beans X-rayed in water or cyanide with *nitrogen* bubbling. ($KCN = 5 \times 10^{-4}$ *M*; 600 r X-rays at 43.5 r/min). The beans treated with cyanide ceased growth completely and did not recover. Those treated in water did not cease growth. These results are repeatable. Irradiation in the presence of cyanide and nitrogen has always led to a markedly greater inhibition of growth than irradiation with nitrogen alone (no cyanide). The strengths of the cyanide solutions and the X-ray dose used in these other experiments were as follows:—

(1) 1.25×10^{-4} *M* KCN; 300 r X-rays (41.7 r/min)
(2) 5×10^{-4} *M* KCN; 300 r X-rays (42.5 r/min)
(3) 5×10^{-4} *M* KCN; 600 r X-rays (62.3 r/min)
(4) 5×10^{-4} *M* KCN; 500 r X-rays (61.6 r/min)

It appears, therefore, that cyanide increases the inhibition of growth caused by X-rays in nitrogen, but does not do so in the presence of oxygen.

In the two experiments (3) and (4) above, the growth of the beans was also measured on the day of treatment. It was then noticed that there was a significant inhibition of growth within 24 hours of the irradiation of the roots in cyanide (see Fig. 5). This inhibition occurs too soon after treatment for a complete mitotic cycle to have occurred. It seems possible that this inhibition may be the result of damage to some system controlling cell elongation.

The cytological experiments.—Beans treated with cyanide (5×10^{-4} *M*) or

water were irradiated with 250 r X-rays (53.0 r/min) with *oxygen* bubbling. The results are shown in Table I. The combination of treatments greatly reduced the number of anaphases available for analysis. There was no evidence of an increase in the number of cells with abnormal anaphases in the roots of beans which had received cyanide treatment.

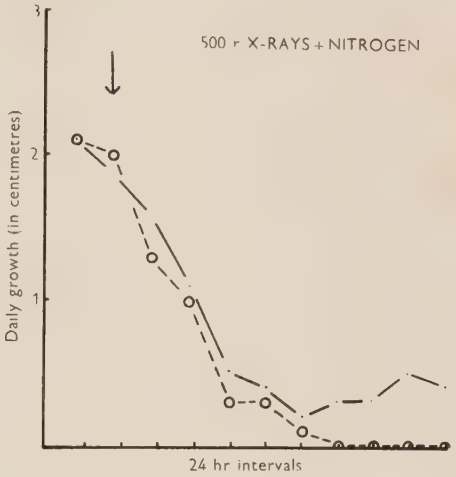

Fig. 5.—Daily growth increments of beans irradiated with 500 r X-rays (61.6 r/min) during treatment with 5×10^{-4} potassium cyanide (○----○), or water (·————·), in the presence of nitrogen.

In contrast, when beans treated in water or cyanide solution (5×10^{-4} *M*) were irradiated with 300 r X-rays (53.0 r/min) with *nitrogen* bubbling, the number of abnormal anaphases was significantly higher in those roots which had received cyanide treatment than in those treated in water (Table II). The results of this experiment were tested by a χ^2 analysis (Table III). The probability level for the differences between the cyanide and water treatments is < 0.001. As might be expected there are significant differences between aberration frequencies at fixation times. The differences between replicates are not significant.

Kihlman, Merz and Swanson [8] have recently confirmed that the increase of aberrations due to cyanide treatment given in conjunction with X-rays occurs only in experiments conducted with nitrogen.

The mitotic index counts from beans irradiated in oxygen with 250 r X-rays (53.0 r/min) show that there is slightly more inhibition of mitosis after cyanide treatment than after water treatments. Nevertheless, both treatments result in a great inhibition of mitosis.

The mitotic index counts of beans irradiated with 500 r X-rays (53.0 r/min) in nitrogen reveal that there is a marked mitotic inhibition following

TABLE I. *Oxygen and 250 r X-rays.*

(a) and (b) are replicate experiments.

Fixation time (time after treatment)	KCN		H_2O	
	Total anaphases	% abnormal anaphases	Total anaphases	% abnormal anaphases
1½	100	56	100	68 (a)
	100	84	100	83 (b)
3	100	55	100	54 (a)
	72	91.6	83	86.8 (b)
6	—	—	29	93.2 (a)
	59	84.7	73	91.8 (b)
9	38	97.3	23	69.6 (a)
	5	100	13	92.2 (b)
12	11	90.9	27	96.2 (a)
	4	75	25	86.8 (b)
16	44	20.4	42	81 (a)
	3	33.3	74	89.2 (b)
24	71	90.2	100	71 (a)
	100	83	9	66.6 (b)
36	63	77.8	100	57 (a)
	100	48	82	70.7 (b)
48	62	67.8	100	59 (a)
	18	44.5	100	73 (b)

cyanide treatment, which is much greater than the inhibition following water treatment.

In summary, beans irradiated in cyanide with nitrogen bubbling show greater inhibition of growth, more abnormal anaphases and more mitotic inhibition than do beans irradiated in water with nitrogen bubbling. When the irradiation is carried out in oxygen there is no evidence that cyanide increases the radiation damage.

The action of cyanide in the presence of α-particles

No growth rate experiments have been conducted with α-particles. In the single cytological experiment made with α-particles, no influence of cyanide upon the numbers of abnormal anaphases could be detected. There was

TABLE II. *Nitrogen and 300 r X-rays.*

(a) and (b) are replicate experiments.

Fixation time (time after treatment)	KCN		H_2O	
	Total anaphases	% abnormal anaphases	Total anaphases	% abnormal anaphases
3	81	54.4	100	44 (a)
	98	72.6	99	56.6 (b)
$4\frac{1}{2}$	41	73.1	22	68.1 (a)
	47	72.4	99	69.6 (b)
6	60	65	55	56.4 (a)
	4	100	100	62 (b)
$7\frac{1}{2}$	45	66.6	48	68.8 (a)
	—	—	23	65 (b)
9	29	82.8	31	64.6 (a)
	18	66.6	50	64 (b)
$10\frac{1}{2}$	11	81.9	100	53 (a)
	100	59	100	49 (b)
12	10	60	40	60 (a)
	17	94.2	100	55 (b)
16	70	67.2	100	57 (a)
	—	—	100	38 (b)
24	100	48	100	40 (a)
	100	60	100	37 (b)

TABLE III. *χ^2-Analysis of the number of abnormal anaphases found after irradiation with 300 r X-rays (53 r/min) in potassium cyanide (5×10^{-4} M) or water, in the presence of nitrogen.*

Source	χ^2	n	P
χ^2 due to times	54.483676	8	v. small
χ^2 due to KCN/H_2O	19.169924	1	v. small
χ^2 due to interaction KCN/H_2O v. times	13.427735	8	0.1
Exptl. χ^2	87.081335	17	v. small
Remainder due to replicates	15.99698	12	0.2
Total χ^2	103.077533	29	v. small

severe mitotic inhibition following all treatments, but the mitotic indices of beans treated with water plus nitrogen, or water plus oxygen, recovered after twelve hours. In beans treated with cyanide plus oxygen or cyanide plus nitrogen, recovery was delayed until twenty four hours after treatment. There was somewhat more inhibition with cyanide plus oxygen treatment than with cyanide plus nitrogen treatment. The dose of radiation used ranged from 17 e to 21 e.

DISCUSSION

The experimental results provide evidence that there is a radiomimetic action of cyanide which is *oxygen* sensitive. Further, cyanide causes an increase in radiosensitivity (the "cyanide effect") when combined with *nitrogen* treatment during radiation. This increase in radiosensitivity did not occur in the presence of oxygen.

These results enable explanations to be offered for the apparent discrepancies between the work of Mottram and Thoday. Mottram did not aerate his beans during radiation, and the author [9] has shown that in similar conditions cyanide increases the inhibition of growth by radiation. Mottram's experiment corresponds therefore to a nitrogen rather than an oxygen experiment. On the other hand, Thoday bubbled oxygen through the solutions during radiation. This prevented anoxia and hence the appearance of the "cyanide effect". The present work thus supports the observations of both Thoday and Mottram, since it indicates that differing oxygen tensions can produce these apparently contradictory results. It is possible that many of the conflicting claims concerning the action of cyanide made by D'Amato and Gustafsson [4], Bacq [2] and Mikaelson [12] could be explained by differing oxygen tensions as well as differences in materials.

Since oxygen and cyanide together cause aberrations, these might be expected to be added to the aberrations caused by the irradiation, thus leading to a greater total yield of aberrations and a greater degree of growth inhibition when roots are irradiated in the presence of both cyanide and oxygen than would result from the action of radiation alone. Such an increase was not observed. In view of the fact that the exposure of roots in oxygenated water to 300 r completely inhibits growth for a period (Fig. 3) it is possible that any additional effect of cyanide might have been missed in the growth rate experiments, but an effect of cyanide should have been revealed by cytological analysis (Table I). No explanations can at present be offered.

Our experiments clearly invalidate the suggestion [15] that the effect of cyanide on radiosensitivity of anaerobic roots is due to a summation of the effect of cyanide and of irradiation.

The possibility that the gaseous atmosphere in our nominally anaerobic experiments was in fact not quite devoid of oxygen, and that cyanide by depressing respiration conserved this small amount of oxygen and thereby raised the sensitivity of the roots to the aerobic level, has to be considered. This possibility seems to be ruled out in the experiments of Kihlman, Merz and Swanson [8] since these authors observed that roots irradiated anaerobically in the presence of cyanide were actually more sensitive than roots irradiated aerobically in the absence of cyanide.

When this work was first reported [10] it was suggested that the radiomimetic action of cyanide, in the absence of radiation, could be due to the accumulation of peroxide, which may be a mutagen. This could be mediated by the inhibition of the cytochrome system, resulting in the aerobic respiration being channelled through the cyanide resistant, oxygen sensitive, flavoprotein systems, which can produce peroxide. The inhibition of catalase by cyanide would also assist the accumulation of peroxide. Kihlman [7] has shown that the increase in the radiomimetic activity of cyanide in conditions of increasing oxygen tension follows a curve which is similar to that which would be expected if enzymes with a low affinity to oxygen such as flavoproteins were involved. Loveless [11], however, claims that peroxide is not radiomimetic in the roots of *Vicia*. Other hypotheses are considered at length by Kihlman, Merz and Swanson [8].

Various hypotheses have been considered to account for the increase in radiosensitivity in the presence of cyanide with nitrogen. The first hypothesis was that cyanide conserved intracellular oxygen by the inhibition of respiration. As mentioned above, this hypothesis is probably excluded by the results of Kihlman *et al.* [8].

A further hypothesis is that cyanide causes an accumulation of peroxide, by the inhibition of catalase. There is evidence that in some conditions irradiation makes nucleic acid more sensitive to peroxide [1, 3]. It is possible that in the oxygen experiments a high concentration of peroxide would be formed physically by the X-rays. The presence of cyanide might not then be of importance. However, in the nitrogen experiments there would be little physically formed peroxide, so that the conservation of this together with any metabolically formed peroxide would be of great importance. While this hypothesis would account for the differences between the cyanide and water experiments in nitrogen, it does not explain the fact that irradiation under

anaerobic conditions in cyanide is more effective than irradiation aerobically in the absence of cyanide.

Kihlman *et al.* [8] suggest that the heavy metal complexing action of cyanide causes the increase in radiosensitivity as well as the radiomimetic effect. In support of this they report an increase in radiosensitivity in experiments conducted under anaerobic conditions, with another heavy metal complexing agent. This agent (cupferron) causes the same type of chromosome break as cyanide when used in the absence of X-rays, but unlike cyanide, this action is not facilitated by the presence of oxygen. While there are difficulties in explaining all the observed results, it seems possible that the cyanide effect may be due to the complexing action of cyanide on heavy metal components of the chromosomes. In the present work, no cyanide effect could be distinguished in the single experiment with α-particles. However, if the efficacy of α-particles approaches a certainty of causing a break, then it would not be possible to detect an increase in the efficacy of α-particles due to the presence of cyanide.

If the action of cyanide is indeed that of a complexing agent, then there may be grounds for the reappraisal of some of the roles of oxygen during radiation.

SUMMARY

1. An investigation has been made of the radiomimetic and radiomodifying action of cyanide on the roots of *Vicia faba*.

2. Cyanide treatment alone causes inhibition of growth and mitosis, and an increase in the number of abnormal anaphases. These effects are most pronounced in conditions of high oxygen tension.

3. When cyanide is used in conjunction with X-rays, there is an increased inhibition of growth and mitosis, and an increase in the numbers of abnormal anaphases. These effects occur when the experiments are conducted with nitrogen bubbling. In contrast, when the experiments are made with oxygen, cyanide does not increase radiation damage.

4. Cyanide does not appear to modify the action of α-particles on *Vicia* roots.

5. Various hypotheses have been considered in relation to these experimental results.

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SPERMATOGENESIS IN ANIMAL AS REVEALED BY ELECTRON MICROSCOPY

V. SPERMATID DIFFERENTIATION OF *DROSOPHILA* AND GRASSHOPPER

G. YASUZUMI, W. FUJIMURA and H. ISHIDA

Electron Microscope Research Laboratory, Department of Anatomy, Nara Medical College, Kashihara, Japan

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In the course of a study on the history of the insect spermatogenesis, the formation of the nebenkern attracted much interest. The mitochondrial origin of nebenkern has been well established with the aid of the light microscope [6, 14, 18, 20, 21, 26–28, 32], and recently by means of phase contrast and electron microscopy [3, 13, 31]. The nebenkern itself becomes divided into two progressively elongating halves. As the spermatid and axial filament grow in length, the paired nebenkern derivatives are pulled out into axially twisted, ribbon-like structures. Thereafter they become difficult or perhaps impossible to follow in light microscopy. It has been assumed that they form an element of the tail sheath or filament, as in other spermatogenesis.

The chromidial or chromatoid body appearing in germinal cells of testes of *Drosophila* and grasshopper especially in the spermatids appears to play an important, although far from completely understood role in the course of maturation of the spermatids. With this body of information available concerning spermatogenesis of *Drosophila* and grasshopper, it was found of interest to investigate with the electron microscope the fine structure of the body and the relationship between this body and other organelles. Zujtin [45] has described the chromidial body, and it is possibly what Huettner [17] referred to as condensed cytoplasmic body. According to Wilson [34], no function is yet known for the chromidial body, but where it has been closely studied it proves ultimately to be eliminated when cytoplasmic detritus is cast from the maturing sperm. Little has been written of the chromidial body in spermatogenesis of the grasshopper in the recent observations by the electron microscope [3, 13, 31].

The present work was planned in order to study the fine structure of the chromidial body and the fate of the nebenkern, which was made in connection with a study of the fine structure of the nucleus in the spermatid of the grasshopper [38–40]. This study revealed also the presence of a characteristic

pattern of organization in the insect spermatids which so far has never been observed. The description of this structural pattern and the discussion of its functional significance are the topics of this paper.

TECHNIQUE

Fresh testes *in situ* of *Drosophila melanogaster et virilis* and *Gelastorrhinus bicolor* de Haan were fixed for one or two hours at 10°C in one per cent osmium tetroxide, adjusted with veronal-acetate buffer to pH 7.3–7.4. After fixation the specimens were directly dehydrated, without washing in distilled water, in a series of increasing concentration of ethyl alcohol and embedded in a mixture of 20 per cent methyl methacrylate and 80 per cent *n*-butyl methacrylate. Sections were cut at an estimated thickness of 200–500 Å with a Shimadzu microtome with glass knives. The sections were examined, without removing the plastic, in an Akashi electron microscope model TRS-50, or a Hitachi electron microscope model HU-10, or an electron microscope of the Japan Electron Optics Company model JEM-T4, or a Shimadzu electron microscope model SM-1. Electron micrographs were taken at calibrated magnifications of 3500, 5000, 7000 and 10,000 and photographically enlarged as desired.

OBSERVATIONS

There are no essential differences apparent in spermatogenesis between *Drosophila melanogaster et virilis* and *Gelastorrhinus bicolor* de Haan, so that a combined description of them should suffice. Moreover, the fine structure of the seminiferous epithelium was so clearly shown in the electron micrographs that it is deemed superfluous to accompany them by lengthy descriptions. In describing the differentiation which the spermatid undergoes, there are at least five principal structures to be noted, the nucleus, the ground cytoplasm, the nebenkern, the chromidial body, and the Golgi complex. Other important structures appear later and will be mentioned as they occur. As to the differentiation and fine structure of the spermatid nucleus see reports by Yasuzumi and Ishida [38–40].

The juxtanuclear region of an undifferentiated spermatid is seen to be composed of two groups of organelles such as mitochondria and chromidial bodies (Figs. 1 and 2). An electron micrograph (Fig. 1) corresponding to this stage shows circular, oval or elongated profiles of sectioned mitochondria in the cytoplasm consisting of very fine uniformly distributed granules of moderate density. Among these profiles there are some that are V-shaped, cucurbit-shaped or duplicated. These bizarre profiles seem to have been fused end to end, and side by side, as indicated by De Robertis *et al.* [13]. The round or oval shaped mitochondria are limited by a well defined limiting membrane, but some elongated mitochondria show both their ends ill

defined. In the spermatid the arrangement of the mitochondrial crests differs from that encountered in the spermatogonia and nutritive cells. The mitochondria are no longer filled up with regularly arranged lamellae or bridges but contain a highly irregular array of crests. These are often distributed more or less at random, being isolated from the limiting membrane in the matrix.

The chromidial body about 0.1–0.2 μ in diameter consists of a dense concentric laminated structure. The ground cytoplasm of the spermatid appears to be composed of a homogeneous substance in which are dispersed a large number of fine opaque particles having a diameter around 60 Å (Fig. 2). The progressive lengthening of the spermatids has now shifted the main mass of the cytoplasm to the distal part, leaving only a thin layer covering the nucleus. The chromidial bodies migrate along the peripheral margin of the cell with development of the nebenkern derivatives, so that a large number of the chromidial bodies appear in the longitudinal sections through the elongated nebenkern derivatives (Fig. 8). However, they are not found in the longitudinal sections through the tail axial filaments, because the tail axial filaments develop through the central portions of the cytoplasm (Fig. 7).

After the mitochondria have been localized to the juxtanuclear region of the spermatid, they are agglutinated into a single body irregularly at first, but tending to become a spherical or an ovoid nebenkern (Fig. 3). The section through the nebenkern illustrates in the outer region large mitochondrial profiles that become less dense by the disappearance of almost internal bridges and matrix still surrounded by a well defined membrane. In the internal region of the nebenkern all mitochondria fuse tightly, showing a dense appearance as compared with the peripheral zone. In the internal region the boundaries of the mitochondria are still defined and the mitochondria maintain their original size. The spherical or ovoid nebenkern is moietied into hemisphere-shaped halves, which have already been shown by Yasuzumi and Ishida [38, 40], and then increasingly elongated until they are long tubules, round at the base and tapering at the tip (Figs. 4–8).

The Golgi complex is located to the peripheral portion of the cell at the stage of differentiation of the nebenkern (Figs. 4 and 5). The Golgi complex contains a system of membranes bounding thin, slit-like semi-opaque spaces which are often dilated to vacuoles of varying size. The membranes are

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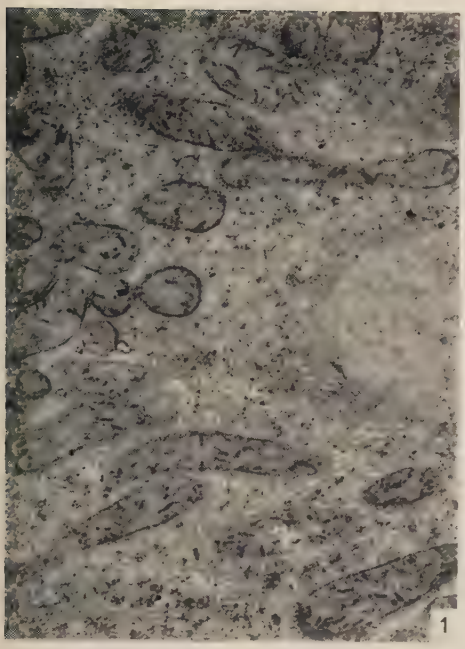
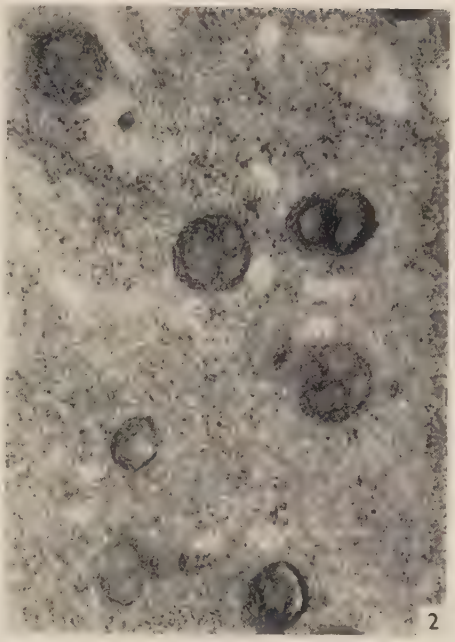
Fig. 1.—Electron micrograph from a thin section through the juxtanuclear region of the early spermatid of *Drosophila virilis*, showing the localization of mitochondria. ×22,500.

Fig. 2.—Micrograph of a number of chromidial bodies appeared in the juxtanuclear region of the early spermatid of *D. virilis*. ×48,000.

Fig. 3.—Micrograph of a fully developed nebenkern of *D. virilis*. ×31,000.





smooth and, at the resolving power available, have no detectable attached granules. No sharply defined borders or membranes demarcating the Golgi zone from the surrounding cytoplasm have been observed.

A peculiar structure, not previously described, is seen in cross sections and longitudinal sections through the postnuclear region of the elongated spermatids (Figs. 4–6 and 13). The conspicuous cytoplasmic components are represented by two to four layers of membrane-bound profiles that circle a pair of elongated nebenkerns and tail axial filaments. Their limiting membranes measure about 10 mμ in thickness, and consist of double layers which are separated by a light space 5 mμ or less in width. Branching profiles and anastomoses between profiles of adjacent rows are seldom encountered. It is a noticeable fact that the cytoplasmic membrane-bound apparatus is associated with the Golgi complex, the chromidial body and the nebenkern. In the longitudinal section the membrane-bound elements appear in parallel arranged layers which are more or less regularly spaced at intervals of 12 to 18 mμ (Figs. 6 and 13). In the remarkably elongated grasshopper spermatids in rod-shape, the membrane-bound cavity profiles in an irregular shape are found surrounding both the tail axial filaments and the paired nebenkern derivatives occupied by a dense substance (Fig. 12). In the nearly mature spermatids of *Drosophila*, the clusters of fine vesicles about 18 mμ in diameter, each having a light core, appear in the cytoplasm between the paired nebenkern derivatives (Fig. 10).

The first change in the matrix of the elongated nebenkern that we were able to find is a relatively sudden appearance of a dense granule 50 mμ in diameter, lying close to the tail axial filaments (Fig. 9). The process of formation of the dense granule continues until the nebenkern becomes transformed into a dense thread-like body. Fig. 10 shows a midway stage in which the matrix lying close to the axial filaments has partially become denser. Figs. 11 to 13 show the nebenkern derivatives entirely composed of the dense substance. The bundle of tail axial filaments is first situated between two divided nebenkern derivatives (Figs. 4 and 5), but as the differentiation proceeds it has migrated outwards from its central position (Figs. 9, 10 and 12). In the spermatids of *Drosophila melanogaster* the dense substance appears only in one of the paired nebenkern derivatives (Figs. 10 and 11), but in *Drosophila virilis* and grasshopper it appears in both in oval shape in cross section

Fig. 4.—A cross section through the spermatids of *D. virilis*, in which a pair of nebenkerns (*N*) and the tail axial filaments (*F*) are developing. The Golgi complex (*G*), the chromidial body (*B*) and the cytoplasmic membrane-bound apparatus (*M*) are clearly visible. The double membrane structure can be seen at the points marked by the arrows. ×70,000.

G
B
N
F
M
N
G
4

(Fig. 12) and in dense thread-form in longitudinal section (Fig. 13). One of the paired nebenkern derivatives in *Drosophila melanogaster* is gradually shrunk and finally scarcely visible (Fig. 11), but in *D. virilis* and grasshopper they are fused into a helical sheath (Fig. 16).

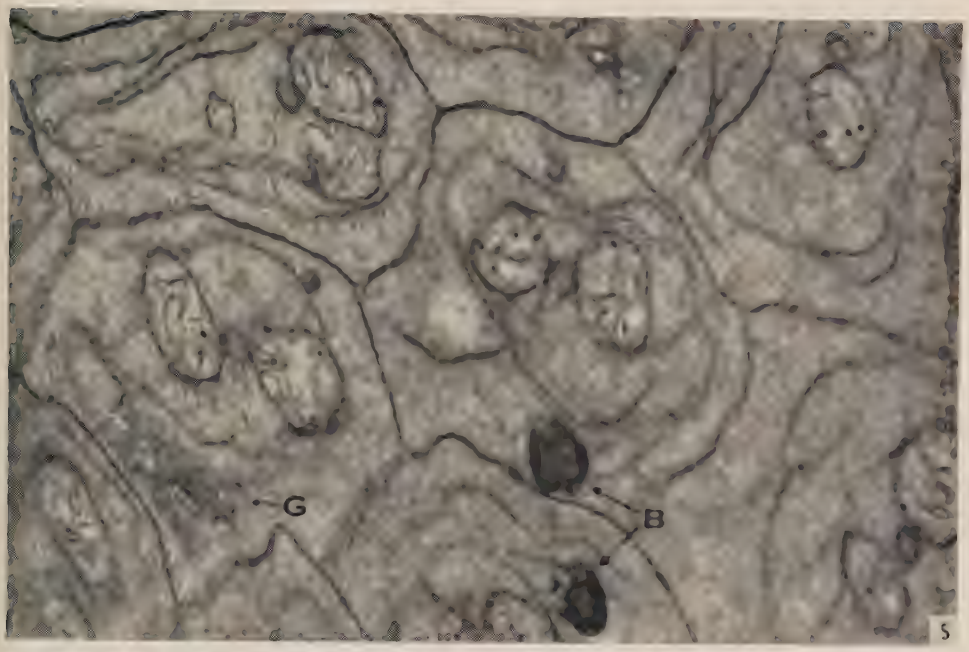
Satisfactory micrographs of nearly mature and mature sperm tail flagella have been obtained and some of them can be seen in Figs. 15–17. The bundle of tail axial filaments is enclosed with a loosely running helical sheath in the middle part of the tail flagellum (Fig. 15), but the distal part of it is devoid of the dense sheath (Fig. 16). The proximal part of the tail flagellum is provided with the sheath with low density, which originates from the nebenkern (Fig. 17).

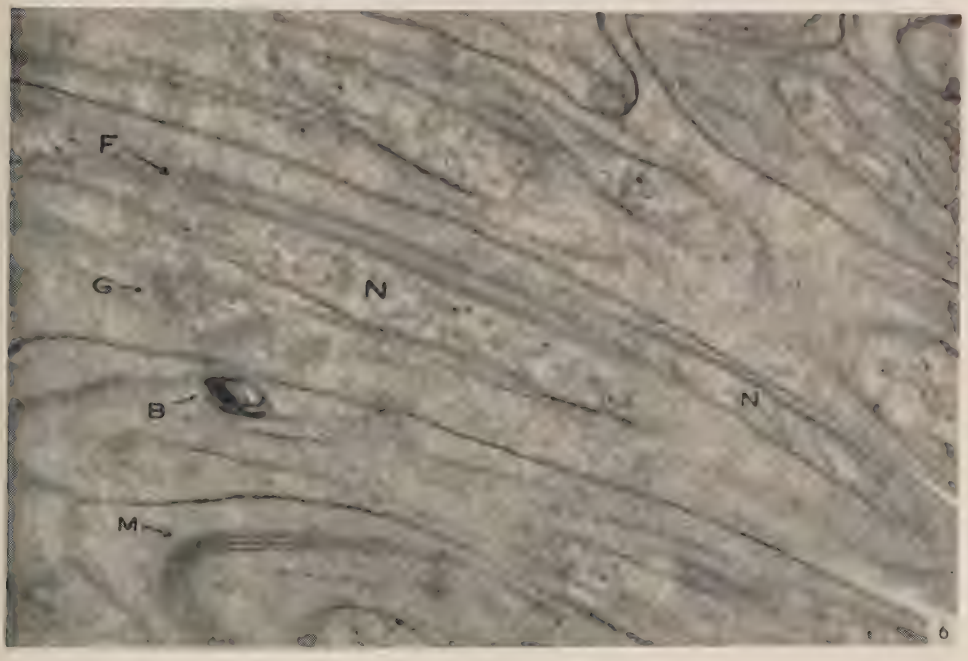
The nearly mature spermatid nucleus of grasshopper displayed a honeycomb structure in a cross section and an array of parallel lines in a longitudinal section [38–40]. However, such a conspicuous structure is scarcely visible in the elongated spermatid nucleus of *Drosophila virilis et melanogaster* (Fig. 17). The nucleus is enveloped with a well defined double membrane.

In cross sections of sperm-tails of the albino rat and mouse, Yasuzumi [35, 36] has first revealed that the tail axial fibrillar component comprises both central and peripheral sets, the former is composed of nine (single or paired) filaments surrounding a pair of filaments, and the latter nine fibrils of much greater density. The axial fibrillar component of the developing tail flagellum of *Drosophila melanogaster* consists of two sets: the central set is composed of a pair of filaments ca. 150 Å in diameter surrounded by a bundle of nine peripheral filaments ca. 180 Å in diameter; the peripheral set comprises nine filaments in irregular shape and of much greater density. The bundle of tail axial filaments has an appearance of snow-like crystal on the whole. The axial filaments are enclosed by a thin membrane (Fig. 10). In a higher magnification of the developing sperm tail flagellum of grasshopper, the central set consists of a pair of filaments surrounding by nine pairs of filaments, each of which about 60 Å in diameter with a light core; the peripheral set comprises dense nine dot-like filaments ca. 150 Å in diameter in a circular array in a cross-section (Fig. 12). The central and peripheral sets of axial filaments can be scarcely differentiated in the nearly mature sperm of *Drosophila* (Fig. 11) and in the mature sperms of grasshopper (Fig. 15).

Fig. 5.—A cross section through the spermatids at the same stage of differentiation as Fig. 4. It displays two chromidial bodies (*B*) and the Golgi complex (*G*). ×40,000.

Fig. 6.—A longitudinal section through the spermatids at the same stage of differentiation as Fig. 4. A pair of nebenkerns (*N*) appear elongated along beside the tail axial filaments (*F*). The chromidial body (*B*) and the membrane-bound elements (*M*) show their profiles. ×40,000.


G
B
5


F
G
N
B
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6

DISCUSSION

The foregoing is an account of some limited observations on morphological changes of spermatids of *Drosophila melanogaster*, *D. virilis* and *Gelastorrhinus bicolor* de Haan during the course of their maturation. Many questions regarding the relationship of the structures observed to one another remain to be answered. For the moment the chief point of interest is the demonstration of a cytoplasmic membrane-bound structure of the spermatid, which is associated with the chromidial body, the Golgi complex and the nebenkern. As far as is known, this is the first time a structure of exactly this nature has been noticed in the electron microscope.

The chromidial or chromatoid substance is considered as a specialized part of the basic cytoplasm with a special chemical composition in which the ribonucleoproteins predominate. Caspersson *et al.* [9, 10] demonstrated that cells in active growth contained ribonucleic acid in the cytoplasm. They suggested that the synthesis of ribonucleic acid could be carried out directly in the cytoplasm next to the nuclear membrane. More recent electron microscope studies of ultrathin sections have shown that, in close relationship with the endoplasmic reticulum, there are dense granules of about the size of macromolecules (10 to 30 mμ) [22, 25]. The granules lie upon and between the cisternae, tubules, and vesicles of the reticulum. This granular component probably contains RNA in high concentration and comprises the so called submicrosome fraction [1, 11]. In the early spermatid of the insects, it is characteristic that the chromidial substance takes a spherical appearance 0.1 to 0.2 μ in diameter, with a concentric laminated structure. Palay and Palade [25] showed a whorl-like structure in the cytoplasm of sympathetic perikaryon and Bernhard [4] and Bernhard *et al.* [5] demonstrated the ergastoplasmic lamellar structure in the rat hypophysis adenoma, which seem to show a similar appearance to the chromidial body examined, although there is difference in size between them. Yasuzumi and Tanaka [43] have shown two

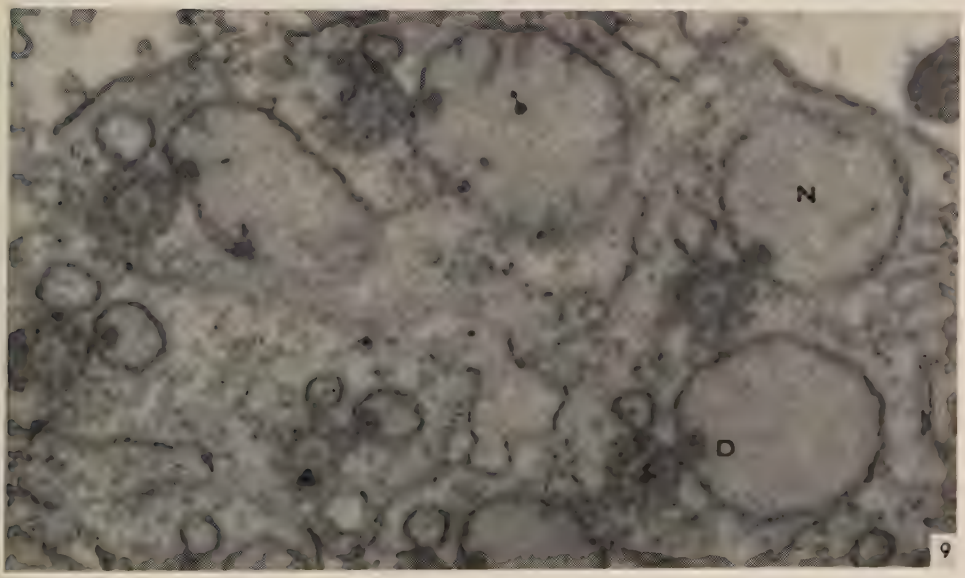
Fig. 7.—A slightly oblique, longitudinal section through the elongated nebenkerns (*N*) and the tail axial filaments of *D. virilis*. ×40,000.

Fig. 8.—A slightly oblique, longitudinal section through the developing spermatids of *D. virilis*, showing the chromidial bodies in the cytoplasm surrounding the nebenkerns and at least four profiles of the nebenkerns. ×22,500.

Fig. 9.—A cross section through the remarkably elongated spermatids of *D. melanogaster* at the stage of appearance of a fine dense granule (*D*) in the matrix of the nebenkern derivative (*N*). The nebenkern derivatives of varying sizes in cross-sections are enveloped with a double limiting membrane and occasionally provided with crests which are displayed with the arrow. The cell membrane becomes often obscure, so that two axial filament bundles seem to appear in a cell. ×60,000.

N
7

8


N
D
9

types of the chromidial bodies of *Cipangopaldina maleata;* one comprises a light substance and a few masses of dense substance, and the other a considerable number of fine granules of intermediate density.

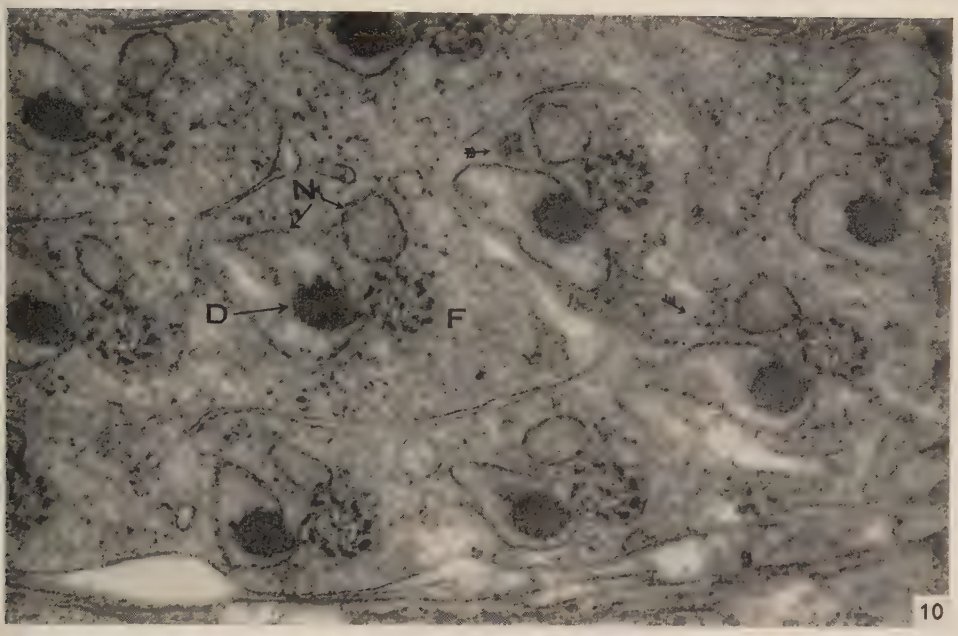
Although Gatenby *et al.* [15] and Beams *et al.* [2] demonstrated the Golgi complex as a duplex structure composed of a group of parallel double-membraned lamellae and a group of associated vacuoles arranged along the compact lamellae in a chain-like fashion in a few invertebrates, the present study has displayed it in tightly but disorderly packed clusters of circular profiles of various diameters, which have no well defined limiting membranes. The Golgi complex is not distributed uniformly throughout the cytoplasm, but occurs in masses in the marginal zone in the elongated spermatid. The Golgi complex is clearly differentiated from the cytoplasmic membrane-bound elements as well as the chromidial bodies.

In all the elongated spermatids examined, a system of membrane-bound elements occurs within the cytoplasm. In sections, this system is represented by more or less parallelly arranged two to four layers profiles, which have no granules either attached to or associated with them. However, this system appears in close association with the chromidial body, Golgi complex and nebenkern in its terminal portions. The membranes limiting the profiles of this system are about 10 mμ in thickness. On the basis of two-dimensional observations, the membrane-bound elements are considered to be broad lamellae each of which comprises a double membrane with a narrow space 5 mμ or less in width.

Various connections between the endoplasmic reticulum and other cell structures have been demonstrated so far in a relatively limited number of cases. Yasuzumi *et al.* [37, 42] have demonstrated that a tubule in the carp erythrocyte, corresponding to the endoplasmic reticulum, is in connection with the nuclear membrane, the Golgi complex and the cell membrane. It has been found that the endoplasmic reticulum is a continuous network of membrane-bound cavities permeating the entire cytoplasm from the cell membrane to the nucleus [23, 33]. With the present observation, it is evident that the cytoplasmic membrane-bound apparatus should not be considered a simple cell organ, but rather a complex system in which the nebenkern,

Fig. 10.—A cross section through the spermatids in a seminiferous tube of *D. melanogaster* at the level of the developing tail flagellum, showing the axial fibrillar component of the tail flagellum (*F*), the dense granule growing larger and larger in the matrix of the nebenkern derivative (*N*) and clusters of fine vesicles at the points marked by the arrows. ×51,500.

Fig. 11.—Cross sections of almost mature sperms of *D. melanogaster* at the level of their tail flagella. The tail flagellum consists of the bundle of axial filaments and the dense sheath with half-moon shape in cross section. ×51,500.



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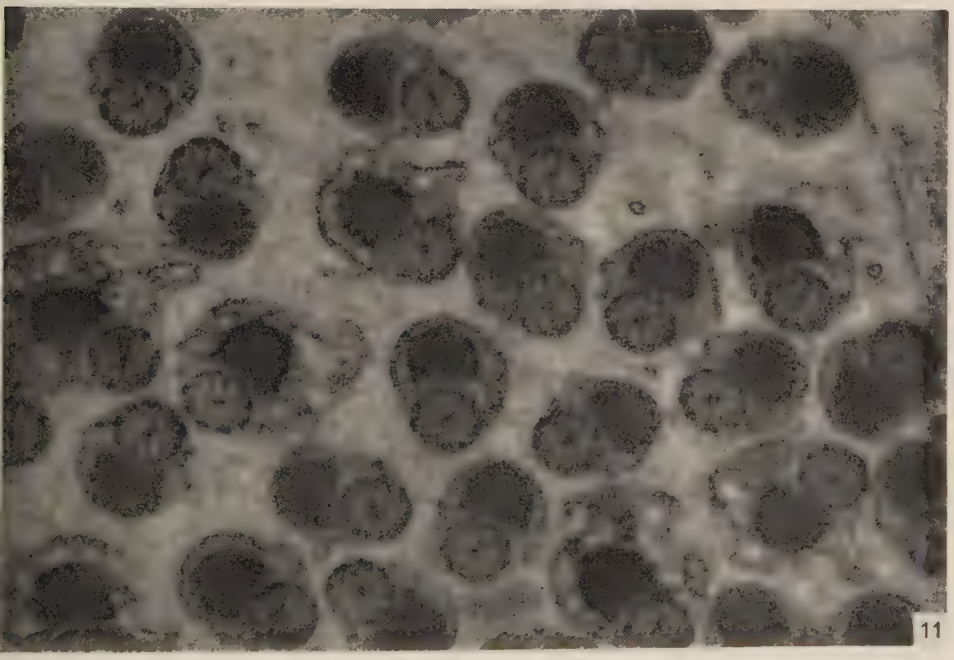

11

Golgi complex and chromidial body are integrated, as Palade has emphasized [24].

We must now compare the membrane-bound apparatus with the cisternae and canals of the endoplasmic reticulum [22, 24, 25, 29]. It was indicated that fenestrations occur in the cisternae of the endoplasmic reticulum and pointed out that there is continuity between the interiors of adjacent lamellae, resulting in a meshwork or network. The present membrane-bound apparatus is composed of two or four layers surrounding the paired nebenkern derivatives, having anastomoses only at a few points. Thus the term "endoplasmic reticulum" with its meaning of a network or meshwork seems inapplicable here, as indicated by Rebhun [30] in the invertebrate oocyte. A further point of difference between the endoplasmic reticulum and the membrane-bound apparatus is the fact that the former has a small particle component upon and between the lamellae, but the latter has a smooth surface, part of which is associated with the chromidial body. However, Palay and Palade [25] have demonstrated that the agranular reticulum of perikarya is similar to structures described in other cells as the Golgi apparatus [12]. The membrane-bound apparatus in the insect spermatid may be a primitive or special form of the endoplasmic reticulum appearing in various mammalian cells.

As the spermatid is remarkably elongated and consequently the paired nebenkern derivatives are filled up with a dense substance, an alteration takes place in the fine structure of the cytoplasm. The membrane-bound apparatus is gradually broken down and is replaced by a pattern of randomly distributed clusters of fine vesicles which can seen be in Fig. 10. This fact has been already revealed by Yasuzumi *et al.* [41] in a series of sections through the elongated spermatids in rod-shape.

The dense granule makes its appearance in the matrix of the nebenkern derivatives in a particular stage of differentiation and grows by accretion of new material to its surface, thus bringing about its progressive enlargement. At the stage of appearance of a dense granule in the matrix of nebenkern, the chromidial body as well as the Golgi complex are scarcely visible

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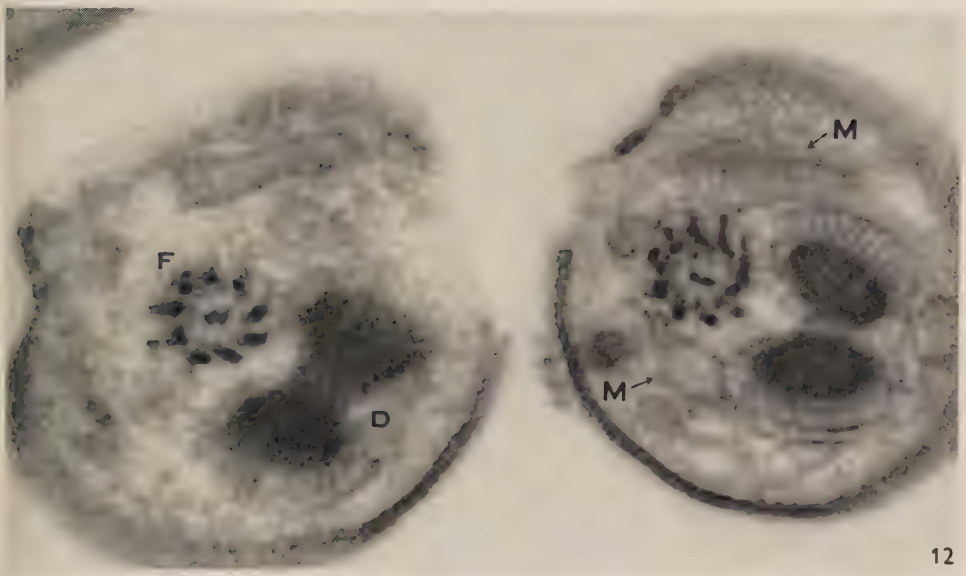
Fig. 12.—Cross sections through two maturing spermatids of grasshopper at the level of developing tail flagellum in a high magnification. The bundle of tail axial filaments (*F*) is composed of two sets, a central set with nine pairs of filaments surrounding a pair of filaments and a peripheral set of nine filaments of much greater density. The paired nebenkern derivatives occupied with the dense substance (*D*) and the dilated membrane-bound cavities (*M*) are clearly visible. ×100,000.

Fig. 13.—Slightly oblique, longitudinal sections through the elongated spermatids of grasshopper at the level of developing tail axial filaments (*T*), showing the nebenkern derivatives occupied with the dense substance (*D*) and the parallel arranged membrane-bound elements (*M*). ×45,000.



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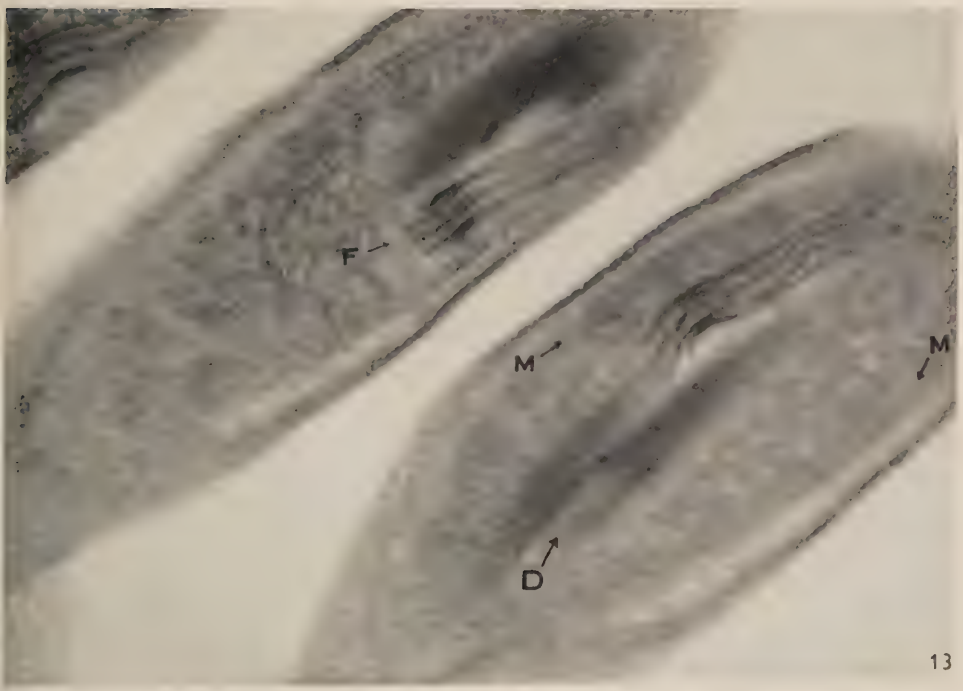


13

in any area of the elongated spermatids examined, but the membrane-bound apparatus is observed in most cases. The dense body which appeared in the matrix of the nebenkern derivatives is indistinguishable from a lipid droplet. Lever [19] has already suggested that a lipid material within the adrenal cortex appears in the mitochondria. In the oocyte of *Cipangopaludina malleata*, Yasuzumi and Tanaka [43] have suggested that the yolk granule and lipid droplet appear in cooperation with the chromidial body and Golgi complex. It is difficult to ascertain whether or not the dense body originates from the matrix of the nebenkern derivatives, the chromidial body, the Golgi complex, or their cooperation.

The connective portion between the middle-piece and the main-piece and that between the main-piece and the end-piece cannot be ascertained, because the tail flagellum of the insect examined is too long to trace by thin sections. By ordinary light microscopy as well as by electron microscopy the tail flagellum appears as a fine dense thread in the smear preparation. However, the middle-piece of the mammalian sperm tail flagellum is enveloped with the helical canalicular sheath, the main-piece by the dense helical tape-like sheath [35], and the end-piece by only the continuous thin membrane 7 mμ in width [44]. Then it is justified that three portions of the tail flagellum of the insects examined (Figs. 15–17) correspond respectively to the middle-piece, the main-piece, and the end-piece.

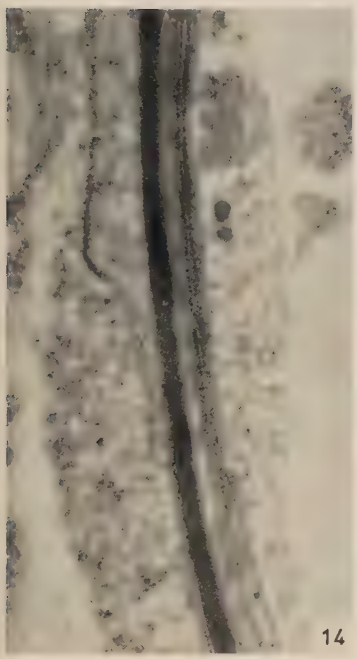
Yasuzumi [36] has shown that the growth period of the helical sheath of the tail flagellum of sperm of *Uroloncha striata* var. *domestica* Flower is divided into two periods, the period for the agglutination of mitochondria, and the period for a sequent transformation into fibrillar elements. In the course of formation of the sheath in the insects is divided into at least five periods: the first period in which the mitochondria are agglutinated into a nebenkern, the next period in which the paired nebenkern halves undergo a peripheral migration, the third period in which a dense substance appears in the nebenkern matrix lying close to the tail axial filaments, the fourth period in which

Fig. 14.—A longitudinal section through the elongated spermatid of *D. virilis*, showing the tail axial filaments and the elongated nebenkerns occupied with a dense substance. ×20,000.

Fig. 15.—A longitudinal section through the end-piece of the tail flagellum of *D. virilis*. ×16,200.

Fig. 16.—Longitudinal section and slightly oblique, cross sections of the tail flagella of sperms of grasshopper. The bundle of axial filaments in the middle-piece of tail flagella is enclosed with a loosely running helical sheath, which is composed of fibrillar elements. ×25,000.

Fig. 17.—Longitudinal sections through the nearly mature sperms of *D. melanogaster*, showing a part of the head (*H*) and the proximal part (middle-piece) of the tail flagellum. The tail flagellum comprises the sheath (*C*) with low density and the axial filaments. The conspicuous structure is scarcely visible in the head. The tail axial filament originates from the portion marked by the arrow. ×24,000.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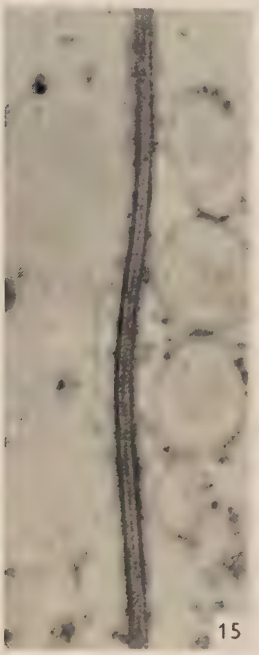
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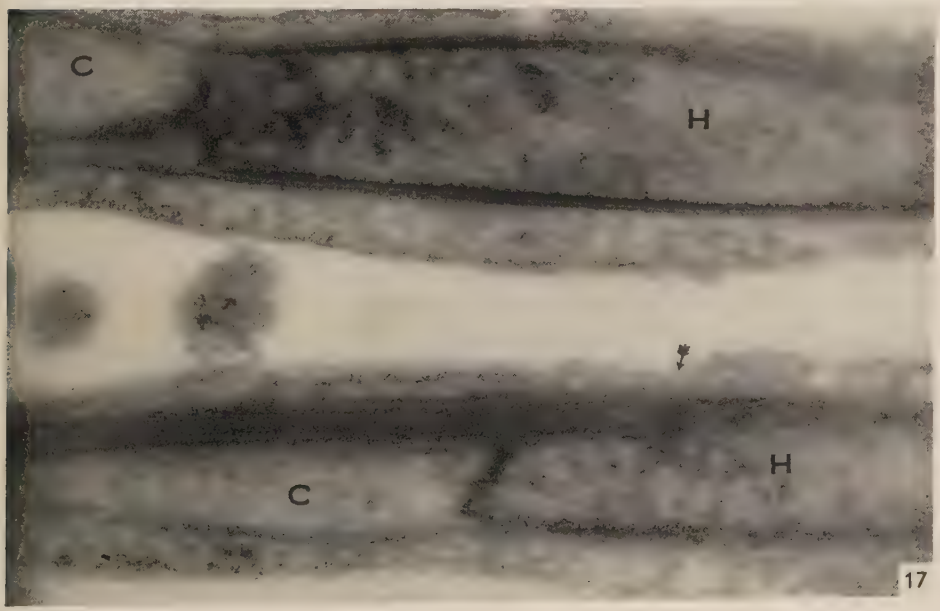
C
H
H
C
17

an accumulation of relatively enormous quantities of the dense material features, and the fifth period in which the nebenkern derivative occupied by the dense substance is transformed into a fibrous helical sheath.

The tail axial filaments are composed of two sets, a central set and a peripheral set, which were first reported by Yasuzumi [35]. The central set of axial filaments in the insect sperm tail flagellum comprises a pair of filaments surrounded by nine filaments (single or paired) which have a core of relatively low density and therefore appear tubular in cross-sections. The peripheral set consists of nine filaments of much greater density. Thus the fine structure of the tail axial filaments of the insect sperm is fundamentally very similar to that of the mammalian, avian, and mollusc sperms [7, 16, 35, 36]. However, it has been demonstrated that the tail axial filaments of such sperms as the toad [8] and the carp [36] are devoid of the peripheral set.

SUMMARY

A systematic study of differentiation of the spermatids in *Drosophila melanogaster et virilis* and *Gelastorrhinus bicolor* de Haan was made with the electron microscope.

The chromidial bodies with a concentric laminated structure, possibly related to the cytoplasmic membrane-bound apparatus, have been found in the juxtanuclear region of the undifferentiated spermatid.

At a particular stage in the evolution of spermatid, namely after the caudal migration of the paired nebenkern derivatives, a conspicuous membrane-bound apparatus appears in the ground substance of the cytoplasm. This apparatus consists of broad, flattened cisternae 5 mμ or less in width, but no fine granules lie between this membrane-bound system and on the surface of the membrane. However, the apparatus is associated with the chromidial body, the Golgi complex, and the nebenkern derivative.

The dense body appears in the matrix of the nebenkern derivative in a particular stage of differentiation and grows by accretion of new material to its surface, thus bringing about its progressive enlargement. The origin and fate of the dense body have been discussed.

The structure of the insect sperm tail flagellum is fundamentally very similar to that of the mammalian sperms: the middle-piece of the insect sperm is enclosed by the elongated nebenkern derivative; the main-piece by the loosely running fibrous helical sheath; the end-piece is devoid of the helical sheath; the axial filaments consist of two sets, a central set and a peripheral set.

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THE EFFECT OF PRIOR GROWTH CONDITIONS ON THE KINETICS OF ADAPTIVE ENZYME FORMATION IN YEAST[1]

CAROLINE RAUT HEBB and JOAN SLEBODNIK

Detroit Institute of Cancer Research and Wayne State University College of Medicine, Detroit, Mich., U.S.A.

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Normal yeast, when grown anaerobically, exhibits negligible oxidative capacity. When such anaerobically grown yeast is aerated in buffer and glucose, the rate of oxygen uptake by whole cells and the activity of various oxidative enzymes increase. The rates of enzyme formation have been found to increase in a straight line from 0 time or to follow S-shaped curves for the various enzymes studied. In one study, the increases in cytochrome *c* content, and in the activity of cytochrome *c* oxidase and of lactic cytochrome *c* reductase, as well as the QO_2 have been shown to follow first order reaction curves, whereas succinic cytochrome *c* reductase increase followed an S-shaped curve ([15], p. 174; [16]).

In the present study, improvement in the medium and changes in conditions during anaerobic growth produced yeast capable of rapidly synthesizing both cytochrome oxidase and the succinic dehydrogenase complex in a straight line from 0 time, on exposure to air. Anaerobic cells grown under less adequate conditions synthesized the same two enzymes more slowly following S-shaped curves.

Demonstration of the variation in the shape of the adaptation curve with the state of the cells is important because of the interpretations that have been made of the kinetics of enzyme adaptation in an attempt to explain the mechanism of enzyme adaptation. Possible bases for the differences in the rates of enzyme syntheses are discussed.

MATERIAL AND METHODS

Medium #1 [5] contains the following, per liter: 5 g peptone; 2.5 g Bacto yeast extract; 6 ml 50 per cent sodium lactate; 0.29 g $CaCl \cdot 2H_2O$; 0.25 g $MgSO_4 \cdot 7H_2O$; 2 g K_2HPO_4; 6 g $(NH_4)_2SO_4$; and 60 g dextrose.

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For the supplemented anaerobic medium (#2), 6 ml of ergosterol solution and 4 to 6 drops of wheat germ oil are added to each liter of medium #1 before autoclaving. To prepare the ergosterol solution, 100 mg of ergosterol and 22 ml of Tween 80 were dissolved in 95 per cent alcohol by heating, and the volume made up to 50 ml with alcohol. The Tween 80 supplies the oleic acid required for anaerobic growth, in addition to ergosterol. In addition, 100 g glucose are added per liter instead of 60 g [1, 9].

Diploid yeast strain LK2G12 was usually grown by one of two methods. In the first, a 2-liter suction flask containing a liter of medium #1 was inoculated, the flask was alternately evacuated and gassed 6 times with purified nitrogen, and each flask was equipped with a mercury-filled trap. The cultures were grown without shaking for 2 days at 30°C.

In the second method, the same kind of suction flask containing a liter of anaerobic medium #2 was inoculated, the flask was gassed with nitrogen in the same manner, and was shaken overnight at room temperature.

For adaptation one gram of cake yeast was suspended per each 80 ml of a solution of 3 per cent glucose in $M/15$ KH_2PO_4 (approximately 1.4×10^7 cells per ml). The suspension was aerated at 30°C by bubbling air through the suspension or in a few cases by shaking.

Aliquots of 80 ml were removed from the aerated suspension at intervals. The number of cells increased up to 2 times during the adaptation in buffer, but all activities were calculated on the basis of a gram of cake yeast at 0 time. The cells were harvested by centrifuging in the cold and washed twice. A half ml of the pH 7.6 buffer and 1.5 g superbrite glass beads (Minnesota Mining and Manufacturing Company) were added to the yeast pellet and the centrifuge bottle filled two thirds full of 3 mm glass beads. The bottles were shaken at 0–4°C for 45 minutes on a Kahn type reciprocating shaker. At the end of this time over 95 per cent of the cells were broken. The bottles were rinsed with cold pH 7.6 buffer and the suspension was centrifuged for 2–4 minutes at $1600 \times g$ to remove the cell walls. The supernatant was then centrifuged for an hour at $22{,}500 \times g$ or at $197{,}000 \times g$ to obtain a pellet of cell particles. The supernatant was clear except for a fatty layer at the top of the tube. Samples of cell particles were tested as soon as they were obtained, although this involved a delay of from 2 to 3 hours from the time of cell breakage. It was demonstrated that there was no enzyme formation on exposure to air during the preparation of the samples for breakage. However, succinic dehydrogenase activity declined gradually when stored in the cold; about 5 per cent of the activity was lost in the course of 2 hours, 10 per cent after 3 hours, and half when stored overnight. After rapid freezing in dry ice and acetone the preparation retained from 40–60 per cent of its activity.

The succinic dehydrogenase activity of the particles was determined using cytochrome *c* as electron acceptor and thus measuring the activity of the entire succinic dehydrogenase complex. The increase in absorption at 550 mμ as the cytochrome *c* was reduced was measured spectrophotometrically ([15], p. 94). Each microcuvette contained between 0.01 and 0.10 ml of diluted particle suspension in pH 7.6 buffer, 3×10^{-5} M cytochrome *c*, 0.003 M freshly prepared KCN, and 0.03 M or 0.02 M succinic acid in 0.05 M phosphate buffer adjusted to about pH 7.5. The total volume was 0.6 ml. All the solutions were made up in 0.05 M pH 7.6 buffer prepared with

Growth conditions:

Expt.	Medium	Agitation	Wet wt. g/liter	Intensity of cytochrome bands
I[a]	1	−	1.8	+ +
II[a]	1	−	2.1	+ + +
III	2	+	7.0	+ +
IV	2	+	3.0	+ + +
V	1	+	4.7	+ +
VI	2	−	12.0	+ / −
VII	2	+	9.5	+
VIII	2	+	15.0	+ + +

[a] The particles in experiments I and II were centrifuged at 22,500 × *g* for one hour; for the remainder of the experiments, they were centrifuged at 197,000 × *g* for one hour. Only slight additional activity was found in the fraction obtained above 22,500 × *g*. In experiment I, the preparation was stored overnight at 0°C before being assayed for succinic dehydrogenase.

glass-distilled water. The assays were run at room temperature (23°C to 26°C) in an air conditioned room.

The rate of reduction of cytochrome *c* was linear with time and the enzyme activity was determined from the slope of this line. Activity was generally measured using at least two enzyme concentrations to insure that the rate of reaction increased linearly with enzyme concentration and was corrected for the very low rate of reduction in the controls without succinate.

Phenazine methosulfate was also used as a hydrogen acceptor in place of cytochrome *c* to measure the activity of succinic dehydrogenase where transport of electrons to cytochrome *c* was not involved [7, 14].

Samples of the same resuspended mitochondrial preparations used in the succinic dehydrogenase assays were frozen in dry ice and acetone and were also assayed for cytochrome oxidase activity with the spectrophotometric method described by Lucile Smith [17]. The concentration of reduced cytochrome *c* used was 33 μM. The rate constants, for each of the 4 or 5 concentrations of enzyme tested for each time sample, produced a straight line, when plotted against the amount of enzyme.

The nitrogen content of the pellets was obtained by the method of Ma and Zuazaga [11].

RESULTS

The rate of increase in succinic dehydrogenase activity during aeration of anaerobic yeast grown under different conditions and also the conditions used in each experiment are shown in Fig. 1. For ease of comparison, the experiments have been numbered and arranged according to the speed of adaptation.

The slowest rate of succinic dehydrogenase formation as well as the lowest yield of anaerobic yeast occurred in the yeast grown standing in medium #1

(Fig. 1, *I* and *II*); the most rapidly adapting yeast and the highest yields were obtained with yeast that was grown with shaking in anaerobic medium #2 (Fig. 1, *VI*, *VII*, and *VIII*). Shaking alone (in medium #1) increased the growth rate and sometimes increased the rate of adaptation to an intermediate rate (Fig. 1, *V*). Growing under optimal conditions (in the anerobic medium, #2, with shaking), but harvesting earlier during the logarithmic growth phase,

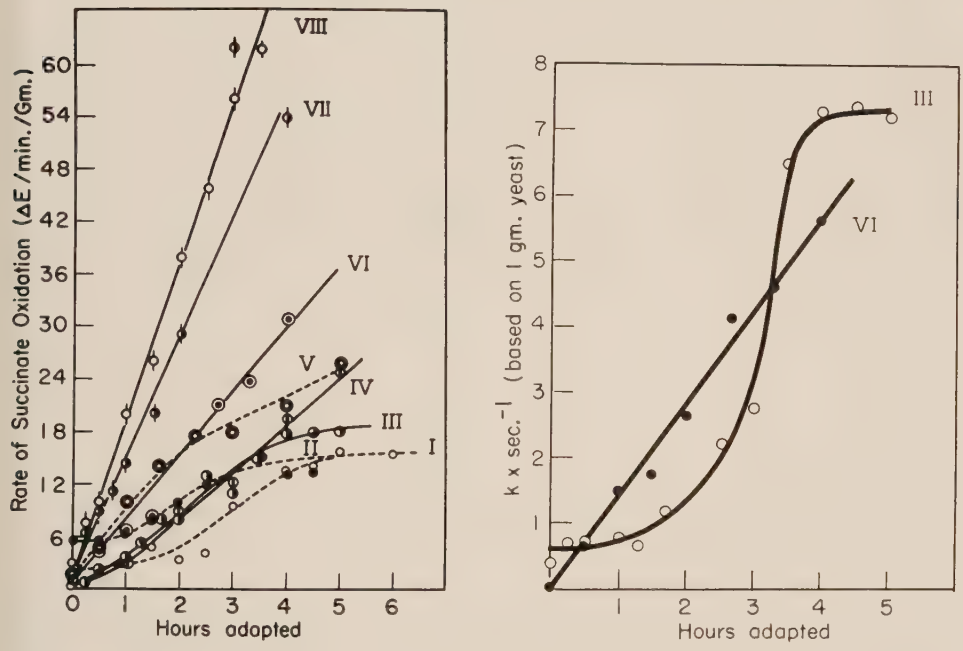

Fig. 1.—Increase in activity of the succinic dehydrogenase complex during aeration of anaerobic yeast grown under different conditions. Each line (I through VIII) represents a different lot of yeast.

Fig. 2.—Increase in cytochrome oxidase activity during aeration of two different lots of anaerobic yeast grown under different conditions. Rapidly frozen suspensions of the particles assayed for succinic dehydrogenase activity in experiments III and VI of Fig. 1 were also assayed for cytochrome oxidase activity.

also resulted in intermediate rates of adaptation (Fig. 1, *III* and *IV*). Comparison of the rates of adaptation with the yields indicates a general correlation between the amount of growth and the rate of adaptation.

Adapted and unadapted anaerobically grown yeast was also assayed for succinic dehydrogenase activity using phenazine methosulfate as hydrogen acceptor, so as to eliminate any effect of the electron transport chain from the dehydrogenase to cytochrome *c*. The increase in activity following adaptation

TABLE I. *Mg nitrogen in particles from yeast samples.*

Each nitrogen determination represents the total nitrogen in the centrifuged particles from one time sample, each of which contains the equivalent of one gram cake yeast at the beginning of aeration. In experiments I and II the cell-free preparations were centrifuged at 22,500 × *g* for an hour; in experiments VII and VIII, they were centrifuged at 197,000 × *g* for an hour.

Minutes of aeration	Experiment			
	I	II	VII	VIII
0	1.57	0.77	4.98	4.33
15			5.45	4.77
30	2.02	1.47	7.74	4.26
45			7.20	
60	1.43	1.74	5.95	4.77
90	1.45	1.97	5.26	4.77
120	1.75	2.11	5.87	4.41
150	1.66	2.36		4.63
180	1.72		6.90	4.77
195		1.81		
210	1.70			4.70
225		2.10		
240	1.66	0.98	7.07	5.87
270		1.22		6.48
300	2.45	2.04	4.74	5.43
360	1.44			

obtained with this assay method indicated that the increase in succinic dehydrogenase activity was not attributable solely to the removal of limitations in electron transport.

A comparison of the increase in cytochrome oxidase activity of fast-frozen suspensions of the same mitochondria that were assayed for succinic dehydrogenase in two experiments, III and VI, is shown in Fig. 2. When the anaerobic yeast was grown to completion of growth under optimal conditions (Fig. 2, *VI*) cytochrome oxidase increased rapidly and in a straight line from 0 time. Where the yeast was grown under the same conditions but harvested during the log phase (Fig. 2, *III*) cytochrome oxidase exhibited a marked lag followed by an autocatalytic increase as compared with the intermediate rate and possible lag in the synthesis of succinic dehydrogenase (Fig. 1, *III*). Thus, the lag and S-shaped adaptation curve can be eliminated for both succinic dehydrogenase and cytochrome oxidase formation by alteration of the growth conditions of the yeast prior to its adaptation.

All the anaerobic yeast was examined with a hand spectroscope at the time of harvest and was found to exhibit the typical anaerobic absorption bands at around 580–590 mμ and 550–565 mμ [3]. The intensity of the bands, as estimated visually, is indicated in Fig. 1; there is apparently no correlation between intensity of the anaerobic bands and the rate of adaptation. In addition, the resuspended pellet in experiment VI was examined quantitatively by Dr. Aristid Lindenmayer in the laboratory of the Johnson Foundation for Medical Physics and found to contain the typical anaerobic spectrum. Thus, incompletely anaerobic cells cannot account for the rapid increase in enzyme obtained in some experiments. The bands were examined with the hand spectroscope during the course of the adaptation and found to shift to the typical aerobic bands at 605, 565, and 550 mμ [3].

In order to determine whether there was sufficient increase in the protein content of the pellet to account for the increase in enzyme activity, the nitrogen content of the pellets from several experiments was determined. These data are given in Table I. It is apparent from Table I that increase in pellet nitrogen cannot account for the many-fold increase in enzyme activity.

DISCUSSION

Inasmuch as a high initial activity of succinic dehydrogenase is associated with the slowest rate of adaptation (Expts. I and II) it may be concluded that the rate of formation of new enzyme is not dependent on the amount of active enzyme present at the beginning. Therefore, autocatalytic increase in the enzyme cannot by itself be the basis of the S-shaped adaptation curve in this case.

Increase in the particulate matter sedimenting at either $22{,}000 \times g$ or $197{,}000 \times g$, as measured by the nitrogen content, cannot account for the increase in enzyme content. However, the volume of the upper fluffy layer of the pellet increases several fold during adaptation, and this is more marked in the slowly adapting yeast. This layer has been shown in other experiments to consist of large vesicle walls which exhibit high enzyme activity [6]. Thus, there is some indication of a change in the nature of the particles during enzyme formation, which is not reflected in the total nitrogen content.

Kinetic studies of adaptive enzyme synthesis have been widely interpreted in attempts to determine the mechanism of adaptive enzyme synthesis. Self-reproducing cytoplasmic particles and, more recently, an autocatalytic increase in the number of active templates, have been proposed to account for the autocatalytic increase commonly found in enzyme formation [18]. A

rate limiting organizer which determines a constant rate of enzyme synthesis has been proposed to account for the straight-line increase obtained with some enzyme systems [13]. It has also been proposed that such linear deviations from autocatalytic kinetics may signify the presence of restrictions which prevent the exhibition of the exponential kinetics of which the system is capable [18].

In the present instance, where both straight-line and S-shaped curves have been obtained with the same two enzymes, slower initial synthesis of enzyme occurs in the case of the S-shaped curves. The lag and the initial slow rate of synthesis associated with the S-shaped curves suggest a deficiency of factors which must be built up after exposure to air before rapid enzyme synthesis can take place. Several facts are consistent with this interpretation. (1) The slowest rates of synthesis of succinic dehydrogenase are obtained with yeast grown in the unsupplemented medium, in which the yeast grows very poorly. (2) The maximum amount of enzyme synthesized is less in the slowly adapting yeast grown in unsupplemented medium (Expts. I and II). (3) We have observed an increase in number of lipid granules toward the end of anaerobic growth [6], and Ephrussi *et al.* have observed an increase both in oxidative capacity and in Janus green B staining toward the end of growth of aerobic yeast [4]. Both of these facts suggest the accumulation of factors as cells reach the stationary growth phase which could account for the more rapid synthesis of enzymes by anaerobic cells harvested near completion of growth as compared with those harvested earlier.

In addition, Lindenmayer [10] has observed (1) a rapid straight-line increase in QO_2 with adaptation of anaerobic cells grown on fat-supplemented anaerobic medium and harvested in the stationary phase as compared with (2) a slower S-shaped increase in QO_2 for anaerobic cells grown the same way, but harvested earlier in the log phase of growth. Correspondingly, the liquid air spectra of the anaerobic cells suggest the presence of a larger amount of pigment (presumably cytochrome b_1) in the stationary phase anaerobic cells.

Klein [8] has shown that yeast strain LK2G12 grown under anaerobic conditions in medium unsupplemented as to fats contains considerably lower quantities of fatty acids and non-saponifiable fats (largely ergosterol) than when grown under aerobic conditions. He has further shown that both fatty acids and ergosterol increase many times on aeration of such anaerobically grown yeast in glucose-buffer solution, and also that most of the fat is synthesized from the added glucose. He has suggested that a relation exists between the synthesis of lipids and the synthesis of enzymes concerned with

respiration, during aeration, because of the known lipid content of the cytoplasmic granules of yeast.

Recently, purified cytochrome oxidase from pig heart was found to contain 33 per cent lipid [12] and the succinic dehydrogenase complex particles obtained from beef heart mitochondria to contain 60 per cent lipid [2].

We have observed a marked increase in the lipid granules of this yeast during aeration, and also variation in the granules and other structures present in the anaerobic cells grown under different conditions which appear to be correlated with the ability of the cells to synthesize the oxidative enzymes [6].

Although we have also shown that the oxidative enzymes are not attached to the lipid granules [6], the granules may still be involved in the synthesis of the enzymes, or they may compete with enzyme synthesis for precursors.

Undoubtedly many factors are involved in the synthesis of the complex particle-bound oxidative enzymes and this is reflected in the wide variation in rates of synthesis obtained with varying conditions. However, it seems likely that lipid deficiency in the anaerobic cells grown in the unsupplemented medium is at least one factor which may account for the lag and slow initial rate of enzyme synthesis in yeast grown in this manner.

SUMMARY

1. The aeration of anaerobically grown yeast in a glucose-buffer solution induces the synthesis of two enzymes generally associated with mitochondria, the succinic dehydrogenase complex and cytochrome oxidase.

2. Depending on how the anaerobic yeast was grown, both enzymes increase rapidly in a straight line from 0 time, or they exhibit a lag and an initial slow increase following an S-shaped curve.

3. S-shaped adaptation curves for the succinic dehydrogenase complex were obtained with anaerobic yeast grown without shaking in a medium unsupplemented as to fats.

4. Rapid straight-line synthesis of the succinic dehydrogenase complex was obtained with anaerobic yeast grown to completion of growth in an improved medium containing ergosterol, oleic acid and a higher sugar content. When the anaerobic yeast was grown in the improved medium, but was harvested earlier in the growth cycle, intermediate rates of adaptation were obtained.

5. The possibility of fat deficiency in the anaerobically grown cells being a factor in the delay in enzyme formation is discussed and also the relation of these findings to the interpretations of the kinetics of adaptive enzyme formation and the mechanism of enzyme adaptation.

The authors would like to acknowledge the suggestions of Dr. Harlyn Halvorson, who was responsible for initiating this study and also the very helpful advice and suggestions of Dr. Aristid Lindenmayer, particularly as to methods of growth and for his determination of the absorption spectrum of some anaerobically grown yeast. Dr. T. P. Singer has been very generous in helping and advising us as to the succinic dehydrogenase assay.

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THE COMPOSITION OF NUCLEOHISTONE FROM PYCNOTIC NUCLEI

R. VENDRELY, M. ALFERT,[1] H. MATSUDAIRA,[2] and A. KNOBLOCH

Centre de Recherches sur les Macromolécules, Strasbourg, France

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The existence of nucleohistone as a true complex has been questioned. Whether the product isolated really preexists in the cell as conjugated proteins or whether it is produced secondarily by combination between the dissolved substances present at a time in the same cellular extract, simulating a definite compound, is still discussed. However, a number of recent analytical data suggest that nucleohistone is a well-defined complex.

The composition of calf thymus nucleohistone does not vary when it is submitted to a great number of dissolutions and reprecipitations; we have performed this operation up to twelve times without any appreciable change.

Davison and Butler [4] found very constant values for the ratios of arginine and lysine to phosphorus in a number of preparations from individual calf thymus glands.

Crampton, Lipshitz and Chargaff [2] found no differences with regard to comparable salt concentrations between different preparations of nucleohistone made at low ionic strengths and others which had been in contact with strong salt solutions.

On the other hand, Klamerth [7] showed that *in vitro* recombination of DNA and histone yielded complexes, the composition of which depended upon dilution.

Last, the composition of the nucleohistone is the same in the different tissues of the same animal [3], and even in different animal species (fishes, amphibians, birds [8]).

Thus, from an analytical point of view, the nucleohistone should appear as a complex whose composition is rather well defined.

Considering these facts, possible changes in composition of such a complex during physiological or pathological changes in the cell would be of great interest. However, only systems in which such changes occur uniformly in all members of a large cell population can be studied profitably by analytical

[1] Fellow of the John Simon Guggenheim Memorial Foundation, 1957; on leave from the Department of Zoology, University of California at Berkeley.

[2] Present address: Faculty of Medicine, Tokyo University, Japan.

methods. Maturation of sperm cells is one such case in which it was possible to demonstrate changes in the desoxyribonucleoprotein complex of the nucleus [12]. Controlled autolysis of tissues offers another opportunity to study a common and widespread pathological process, namely nuclear pycnosis which results in striking alterations of the cytochemical properties of chromatin [1].

MATERIAL AND METHOD

Our material consisted of calf kidneys. Control kidneys were frozen soon after the death of the animal and subsequently studied. Experimental kidneys were kept aseptically at 37°C for various periods of time and the progress of autolysis was followed histologically on small tissue samples taken at different times. After 24 hours the tissue had undergone very uniform pycnotic changes consisting in striking reduction of nuclear volume and homogenization of nuclear content (compare Figs. 1 and 2). The isolation of nuclei was made with the citric acid method published previously [11].

While the isolation for subsequent analysis of nuclei from normal kidneys is quite easy with the citric acid method, it has proved impossible to obtain clean pycnotic nuclei in the same way: the nuclei remain contaminated by large masses of cytoplasm. A comparison between isolated normal and pycnotic nuclei is shown in Figs. 3 and 4.

While it was thus not possible to compare analytical results concerning the protein from normal and pycnotic isolated nuclei, we have been able to extract nucleohistones directly from both types of whole tissue for comparison. Normal nucleohistone in molar saline solution is very viscous and precipitates in fibrous form by dilution. The nucleohistone from autolysing kidney was not viscous and produced a finely granular precipitate.

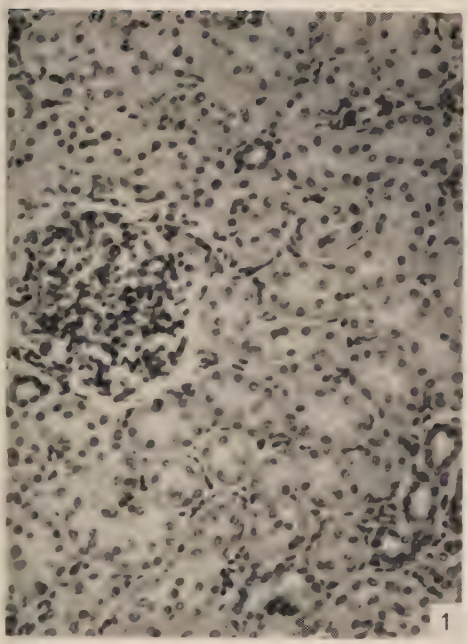
The chemical measurement of DNA was made by the Dische method [5] and arginine was determined by the technic of Dumazert and Poggi [6]. Chromatograms were made from both nucleohistones from pycnotic and normal kidney and from nucleohistone from calf thymus, each chromatogram derived from hydrolysate containing identical amounts of DNA.

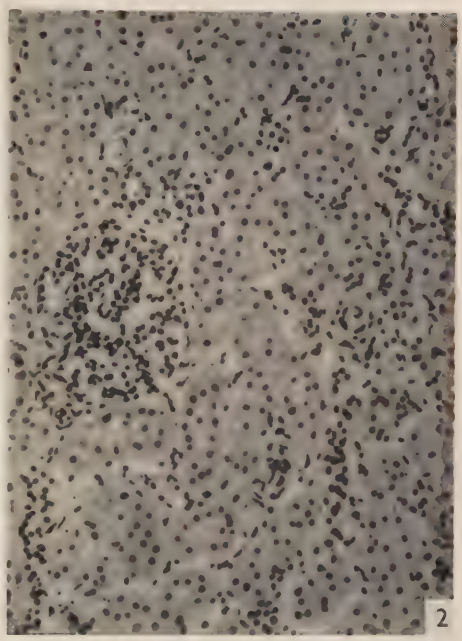
RESULTS

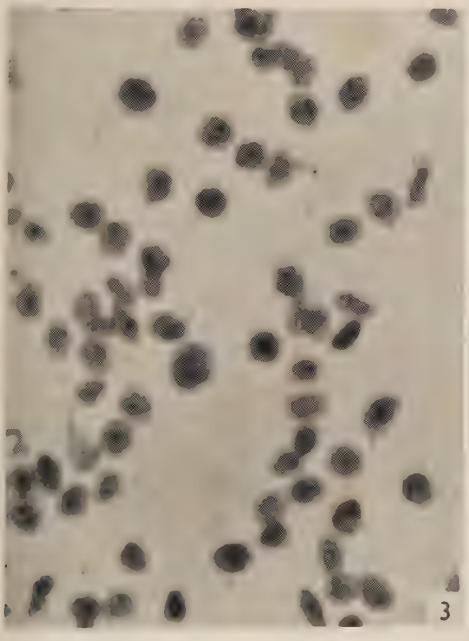
Isolated nuclei.—The only analytical result that could be obtained on the pycnotic material pertains to the DNA content of such nuclei. In accordance with previous cytochemical results, such nuclei were found to contain the same amount of DNA as normal control nuclei.

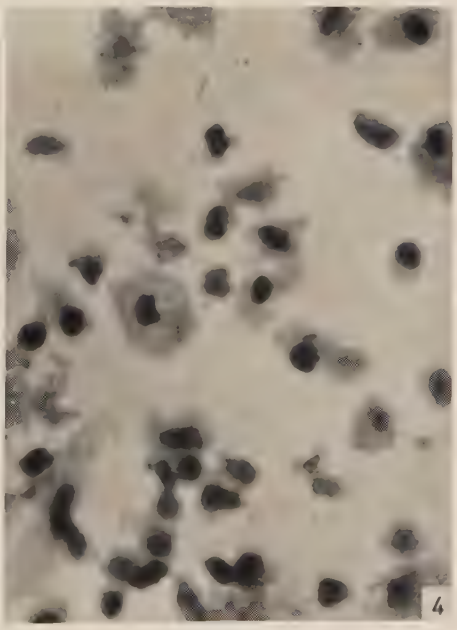
Figs. 1 and 2.—10 μ sections of calf kidney; Feulgen, counterstained with acid Fast green; × 200. *Fig. 1*, control; *Fig. 2*, pycnotic.

Figs. 3 and 4.—Smears of nuclei isolated in $\frac{1}{3}$ *M* citric acid; Feulgen–Fast green; × 640. *Fig. 3*, control; *Fig. 4*, pycnotic.









Nucleohistones.—The nucleohistone isolated from autolysed kidney was depolymerized in contrast to the highly polymerized nucleohistone obtained from fresh kidney. But, the analysis of the two products gives very similar results. The molar ratio Arginine/nucleic P is the same in the nucleohistone of pycnotic kidney nuclei (0.27) as in the nucleohistone of normal kidney (0.24), thymus (0.25) and liver (0.26). The amounts of arginine, lysine and histidine in the 4 histones are quite comparable as can be seen in the Table I.

TABLE I. *Amino acid residue (g) per 100 g protein.*

	Calf thymus histone	Calf liver histone	Calf normal kidney histone	Calf pycnotic kidney histone
Arginine	12.6	11.8	12.3	11.9
Lysine	15.5	15.2	16.1	15.6
Histidine	2.4	2.5	2.3	2.2

The chromatogram of the pycnotic kidney nucleohistone is quite comparable to that of the normal kidney and thymus (Fig. 5).

DISCUSSION

Several points should be stressed as a result of the present investigation:

Previous cytochemical studies [10, 1], have dealt with the DNA content of pycnotic nuclei. As pointed out by Alfert, pycnosis *per se* does not lead to a lowered nuclear DNA content unless the degenerative process is accompanied by nuclear fragmentation which is not the case in the system studied by us. (If autolysis is permitted to proceed beyond the optimal period at which the pycnotic appearance of the nuclei is very uniform throughout the tissue, the nuclei seem to dissolve rather suddenly and large areas within the tissue are entirely devoid of demonstrable nuclear material.)

The whole nucleohistone complex, not just DNA, appears to become depolymerized during autolysis as evidenced by its lack of viscosity in solution and by its granular form of precipitation. Contrary to the theory of Kurnick [9] such pycnotic material has however been found to exhibit *increased* methyl green stainability when compared to normal chromatin [1].

The behaviour and chemical composition of pycnotic nucleohistone are very similar to those of normal nucleohistone. The changed cytochemical properties of pycnotic nuclei, their relatively increased basophilia and acido-

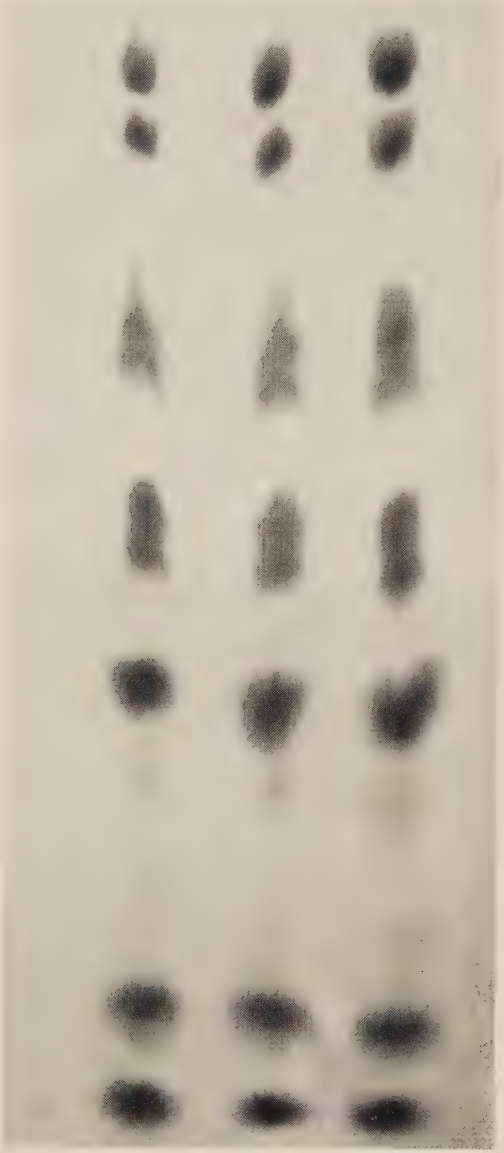


Fig. 5.—Chromatograms of nucleohistones extracted in *M* NaCl from calf thymus, fresh kidney and autolysed kidney; hydrolysates containing identical quantities of DNA were used in all three cases.

philia, must therefore be due to an "unmasking" of stainable groups in the course of autolysis. The present data thus lend support to Alfert's hypothesis on the mechanism of pycnotic degeneration.

The unchanged composition of nucleohistone from autolysing tissue is an

argument in favor of the reality of a preformed well-defined nucleohistone complex. If this complex were only an artefact of the extraction and precipitation procedure, one could hardly expect it to form in identical fashion from such different environments as those represented by normal and autolysing tissues.

SUMMARY

1. The DNA content of pycnotic kidney nuclei is the same as that of normal nuclei.

2. The nucleohistone isolated from pycnotic kidney is depolymerized but retains its properties of precipitation and solubility and can be isolated in the same way as the normal nucleohistone.

3. The chemical composition of nucleohistone of pycnotic kidney is the same as that of nucleohistone of normal kidney though the well-known changes of pycnosis had appeared in the aspect and staining properties of the nuclei in the autolysing tissue. The significance of these results in relation with the results obtained in cytophotometry is discussed.

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NUCLEAR UPTAKE OF LABELLED ADENINE IN THE SEMINAL VESICLE OF THE MOUSE

S. R. PELC

Medical Research Council, Biophysics Research Unit, London, England

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The early investigators of the incorporation of labelled precursors into deoxyribonucleic acid (DNA) assumed that this compound, like others, would show some metabolic activity. Subsequently the low uptake of tracers in non-dividing tissues revealed in later experiments led to the assumption that deoxyribonucleic acid is only synthesised in preparation for cell division or during the formation of polyploid cells and is chemically stable afterwards. The observations reported here indicate incorporation of labelled adenine into the DNA of the epithelial cells of the seminal vesicle of the mouse greatly in excess of mitotic activity. A critical survey of the relevant literature in the discussion shows, however, that there is evidence for significant incorporation of tracers into the DNA of non-dividing tissues at rates which usually are low compared with those found in dividing tissues and which vary widely between organs. It is suggested that cells in some non-dividing tissues synthesize DNA for definite metabolic purposes and that the amount of synthesis depends on their function.

MATERIALS AND METHODS

C_3H or C_{57} black × C_3H F_1 hybrid mice 3 to 5 months old were injected intraperitoneally (i.p.) with 20 or 40 μC of [8-^{14}C]-adenine HCl in 0.4 to 0.5 ml of water. The labelled adenine, obtained from the Radiochemical Centre, Amersham, was dissolved and all animals belonging to a given experiment were injected immediately afterwards.

Organs were fixed for 1 hour in acetic acid–alcohol (1:3) followed by 24 hours in formol saline, 5 μ sections were cut after paraffin embedding. Stripping-film autoradiographs were prepared of sections which had been de-paraffinized in xylol and taken to water through graded alcohols [22]. Others were treated with ribonuclease, 10 per cent perchloric acid at 4°C for 18 hours or 5 per cent perchloric acid at 60°C for 30 minutes. Previous experiments [21] have shown that ribonuclease and cold perchloric acid are effective in removing ribonucleic acid (RNA) whilst hot perchloric acid dissolves both nucleic acids.

Observations were made on autoradiographs stained with haematoxylin and eosin or methyl green–pyronine after photographic processing. Feulgen stained sections were used to verify the presence of dividing cells.

RESULTS

Seminal vesicle (*epithelium*).—In the mouse the seminal vesicle has a folded epithelium surrounding a lumen filled with secretion (Fig. 1). The folds are very close at the fundus of the gland, i.e. at the end furthest removed from the urethra and are wider towards the urethra. Consequently the amount of secretion relative to the number of epithelial cells varies widely within the gland. No differences in the uptake of labelled adenine were found between the different parts of the gland. Some smooth muscle is found surrounding the gland.

In unextracted sections autoradiographs of the seminal vesicle of mice killed 1 day after i.p. injection of [8-^{14}C]-adenine show a fairly uniform uptake of the tracer in the nuclei and cytoplasm of epithelial cells (Fig. 2). Occasionally traces of activity are found in the secretion.

Some nuclei are labelled after extraction with cold perchloric acid; patches of activity are occasionally found in the cytoplasm (Fig. 3). Labelled nuclei show on an average 11 grains per nucleus (Table I), a much higher concentration than that found over similar areas of cytoplasm. No positive nuclei are found after extraction with hot perchloric acid, whilst the cytoplasmic autoradiograph is as weak as it was after extraction with cold perchloric acid.

Nearly all adenine incorporated into the nuclei of cells of smooth muscle is removed by cold perchloric acid (Table I). This would be expected since biochemical work has shown incorporation of ^{32}P into the RNA but very little into the DNA of muscle. The data prove that the loss of nuclear RNA after perchloric acid treatment, as indicated by the staining reactions, is complete in the sense that all RNA adenine is removed. The remaining count of 1.4 ± 0.3 grains per nucleus covering an area of approximately 50 μ^2 is largely due to background.

The histogram (Fig. 4) shows the distribution of labelled nuclei of epithelial cells after the various treatments. Since the sections were cut at a nominal thickness of 5 μ, the diameter of the epithelial nuclei will be ap-

Fig. 1.—Seminal vesicle of the mouse, haematoxylin and eosin. Note the closely folded epithelium with relatively small amounts of colloid in the top half and large area at the bottom. The clear spaces are due to shrinkage.

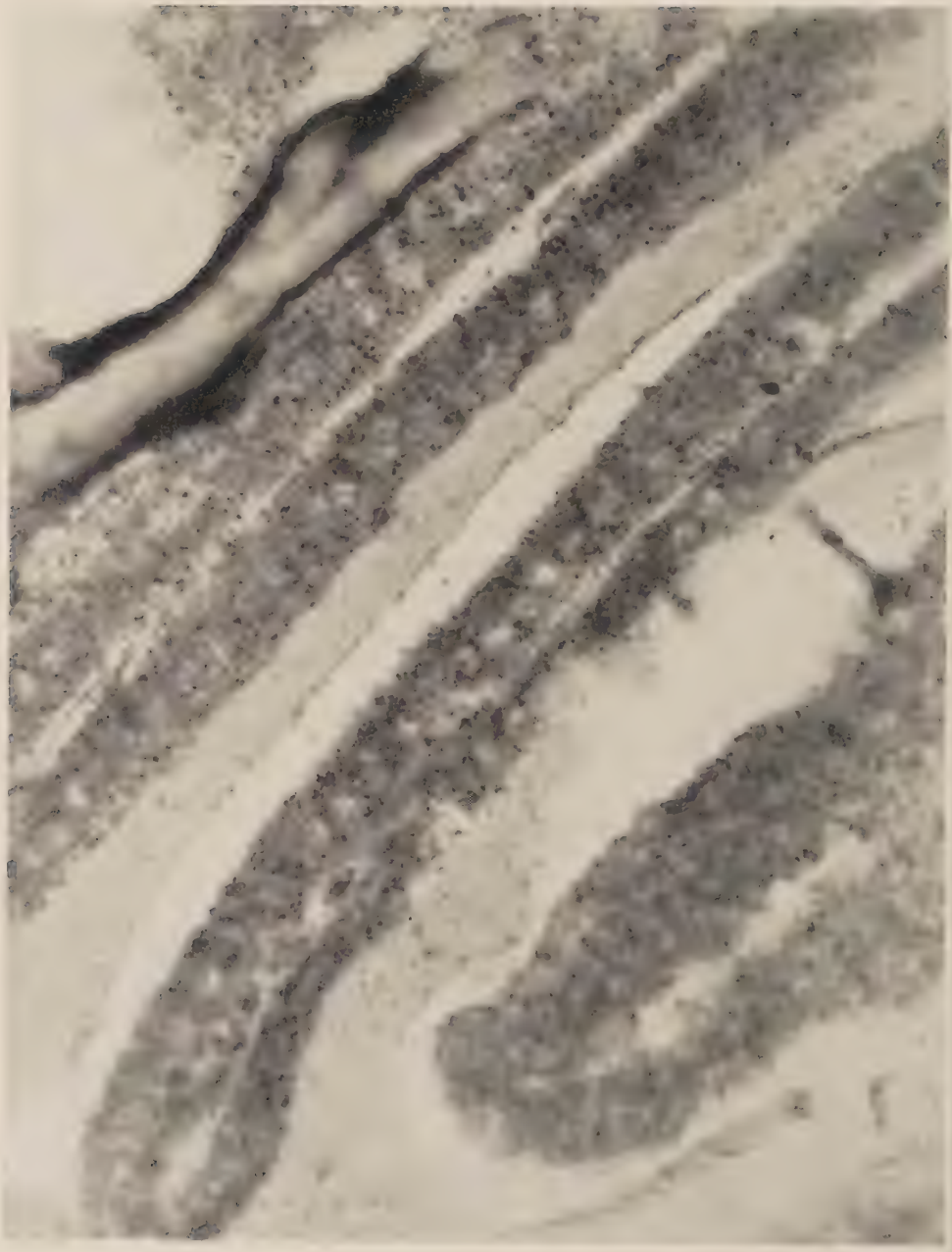

Fig. 2.—Stripping film autoradiograph of the seminal vesicle of a mouse killed 1 day after injection of [8-^{14}C]-adenine. 55 days exposure. No extraction after deparaffinising. Haematoxylin and eosin.

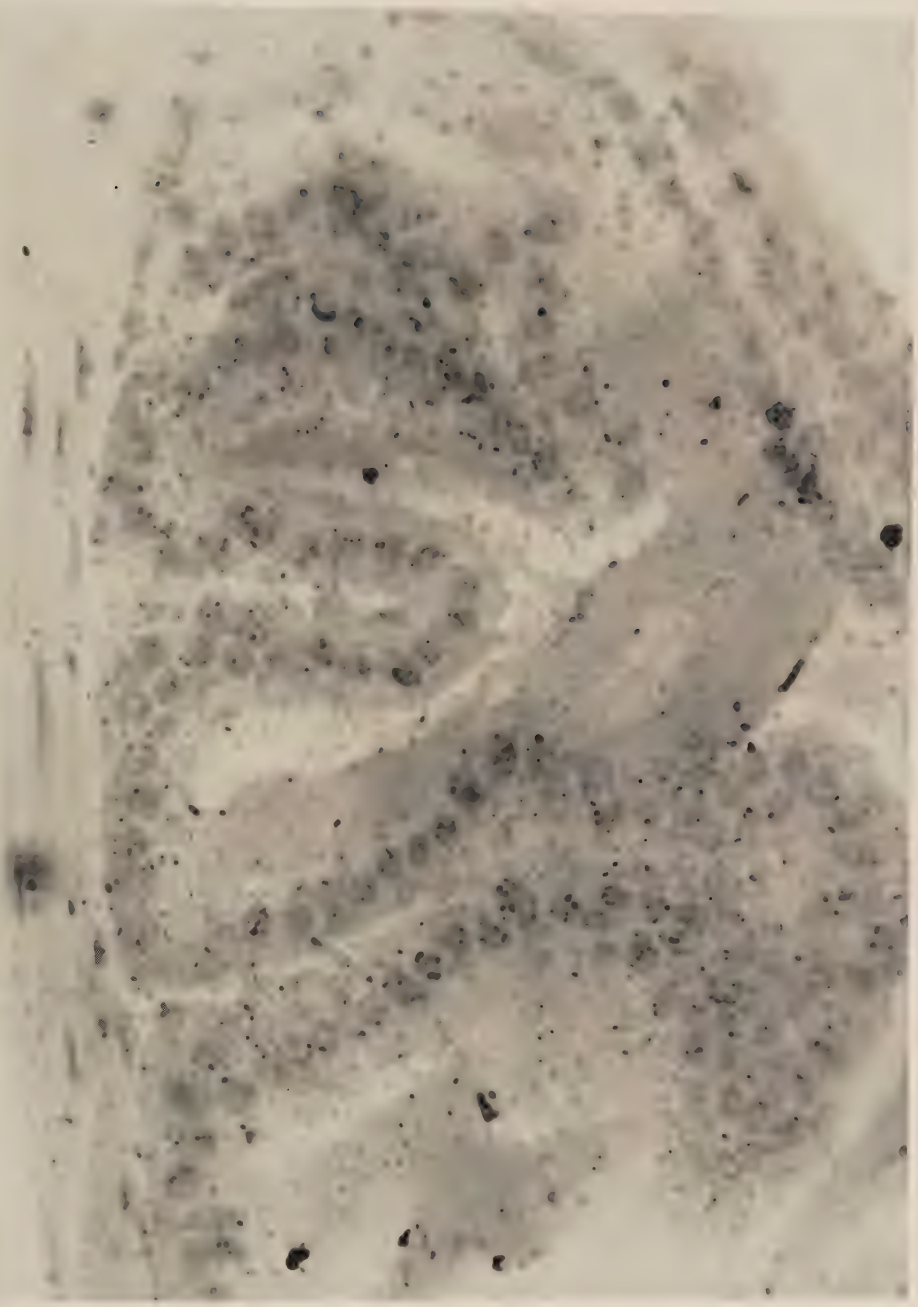

Fig. 3.—As Fig. 2 but extracted with 10 per cent perchloric acid at +4°C for 18 hours after deparaffinising. Note the difference in stainability of the cytoplasm, the decrease in cytoplasmic autoradiograph and the absence of autoradiograph over the nuclei of muscle cells.

TABLE I. *Grain counts on autoradiographs of cell nuclei of the epithelium of seminal vesicle and of muscle.*

(Mice killed one day after injection of [8-^{14}C]-adenine.)

Treatment	Grains per nucleus	
	Seminal vesicle	Muscle
Water	22 ±1.7	7.4±1.1
Cold PCA	11 ±0.9	1.4±0.3
Hot PCA	1.1±0.3	1.1±0.3

PCA = perchloric acid.

proximately equal to the section thickness and the autoradiographs will in many instances be due to parts of nuclei. Nuclei which were obviously only thin slices were rejected for grain counts but it can be assumed that more heavily labelled nuclei would have been found if autoradiographs of whole nuclei had been counted. To avoid this error, it is planned to repeat the experiments, using squash preparations. In spite of these uncertainties, some conclusions can be drawn from the histograms.

All epithelial nuclei were found to be positive in autoradiographs of unextracted sections (Fig. 4) indicating that all nuclei incorporate ^{14}C-adenine during 24 hours; at present it is not possible to state whether all nuclei incorporate equal amounts or whether significant differences in their metabolism exist. After extraction with cold perchloric acid approximately 20 per cent of the nuclei are definitely negative and DNA synthesis during 24 hours is confined therefore to a certain proportion of these non-dividing cells. Forty per cent of the nuclei are well labelled, the remaining 40 per cent which are weakly labelled nuclei cannot be classified since it is unknown what proportion of them represent slices of nuclei. It is possible that a bimodal distribution is smoothed out. All nuclei are unlabelled after extraction with hot perchloric acid. The remaining 1.1 ± 0.3 grains per nucleus (Table I) are compatible with a satisfactory background.

Three days after injection epithelial cells of the seminal vesicle are well labelled in unextracted slides. After extraction with cold perchloric acid many labelled nuclei are seen, the cytoplasm of a fair proportion of the cells is labelled. Little change is observed in animals killed six days after injection. The cytoplasmic autoradiograph after cold perchloric acid is heavier than at 3 days. Both at 3 and 6 days after injection cytoplasmic

autoradiograph approximately equal to that after cold perchloric acid extraction is observed in slides treated with hot perchloric acid. No nuclear autoradiograph remains after this treatment.

Testis and intestines.—The following observations are based on autoradiographs of testes and intestines prepared together with those of the epididymis.

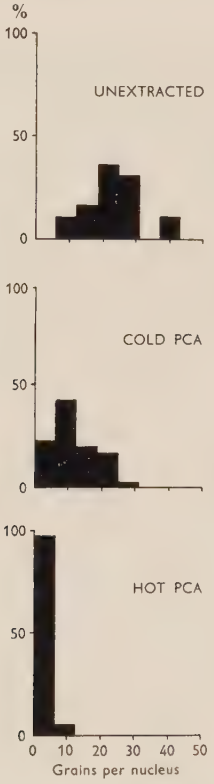

Fig. 4.—Number of grains over nuclei of epithelial cells of seminal vesicle 1 day after injection with [8-^{14}C]-adenine. Based on counts on 75–100 cells.

In most instances the sections of testis, epididymis and seminal vesicle were on the same slide, those of the intestines on a different slide. Both slides underwent all treatments simultaneously.

These observations on testis closely follow those reported previously [21, 22]. After 1 day most spermatogonia are well labelled and spermatocytes are weak in unextracted slides. A small proportion of labelled spermatogonial nuclei remains after extraction with cold perchloric acid but no autoradiograph can be observed after treatment with hot perchloric acid. The percentage of labelled nuclei increases up to 6–8 days.

In the intestine very intensive autoradiograph especially in approximately the lower half of the crypts is observed in unextracted sections of animals killed one day after injection of ^{14}C-adenine. The heaviest autoradiograph is found in the middle third in the small intestine 2 days after injection and near the tips of the villi after 3 days. The stroma-cells enclosed by the villi are labelled.

After extraction with cold perchloric acid nuclear autoradiograph is observed in the regions of heavy labelling as well as a weaker labelling of nuclei in the crypts at times later than 1 day. Relatively heavy cytoplasmic autoradiographs can be observed after treatment with cold or hot perchloric acid, whilst the latter removes all tracer from the nuclei. The stroma cells of the villi are unlabelled 1 day after injection in slides extracted with cold perchloric acid, but many of them are labelled at 6 days and later. Hot perchloric acid removes all tracer from these cells at all times.

Staining reactions.—The epithelium of the seminal vesicle of the mouse is strongly basophilic in preparations not extracted with cold or hot perchloric acid. Nuclei and cytoplasm are almost equally stained with Ehrlich's haematoxylin and eosin and recognition of nuclei is sometimes difficult. The nuclei stain well with haematoxylin after extraction with cold perchloric acid, whilst the cytoplasm shows a clear pink colour with eosin.

Cold perchloric acid causes a great reduction in the uptake of pyronin by the cytoplasm of epithelial cells, after treatment with hot perchloric acid a very weak uptake of pyronin in the cytoplasm can be observed whilst the nuclei are unstained.

As would be expected [21], the staining reactions thus indicate that cold perchloric acid removes all ribonucleic acid and hot perchloric acid removes both RNA and DNA.

No cells in division were seen in Feulgen stained sections of the seminal vesicle and therefore no mitotic counts were undertaken. Allen [2] has found a mitotic index of 42 per 100,000 cells in the seminal vesicles of mice 6 hours after injection of colchicine. The seminal vesicle can therefore be regarded as non-dividing tissue in the sense that during 24 hours only a small number (at the most of the order of 2 in 1000) of cells will prepare for division and that therefore indications of metabolic activity which are found in a considerable proportion of cells are unlikely to be connected with mitosis or preparation for it.

The intensity of Feulgen-staining of the epithelial nuclei in the seminal vesicle appears equal to that of other nuclei which can be assumed to contain diploid amounts of DNA. No giant nuclei were found in this tissue. Pre-

liminary measurements by means of Feulgen photometry confirmed the visual observations [24]. No tetraploid or intermediate values which are usually found in dividing tissues were observed.

DISCUSSION

The results of the staining reactions, autoradiographs of various organs and the grain counts show that [8-^{14}C]-adenine incorporated into the nuclei of the epithelial cells of the seminal vesicle and observed after extraction of the sections with cold perchloric acid, can be regarded as incorporation into DNA. This incorporation is surprising in view of the fact that available evidence indicates that it represents incorporation into the DNA of a non-dividing tissue, whilst at present it is generally held that DNA is stable in such tissues. The evidence quoted in support of this latter view can be grouped as (*a*) biochemical experiments which give very low values for the specific activity of DNA after application of labelled precursors in non-dividing tissues as compared with dividing ones, and (*b*) the finding that in various tissues the amount of DNA per cell is found to be twice that of haploid cells.

Most of the available biochemical data on DNA synthesis in non-dividing tissues which seem relevant here are based on tracer experiments in liver, kidney, muscle and brain. Many dividing tissues are composed of dividing and non-dividing cells and for technical reasons the relative proportion of DNA synthesis which leads to mitosis and that which does not cannot easily be determined by biochemical means in such materials; the available experiments are reviewed below. It should be emphasised that experiments on dividing cells which are by some means prevented from dividing may not give conclusive evidence on the question of DNA synthesis in non-dividing tissues, since inhibition of mitosis does not necessarily entail differentiation.

Excellent summaries of the literature on the incorporation of various labelled precursors into the DNA of various organs have been presented by Davidson [10] and Smellie [25]. It has been found that ^{32}P is incorporated into the DNA fraction of normal liver at a rate of approximately 1/20th of that of the intestine. Stevens *et al.* [28] estimated that 42.8 per cent (or 52.7 per cent if allowance is made for the time-lag in the action of colchicine) of the cells in the intestinal mucosa are renewed daily. If the observed incorporation of ^{32}P into liver DNA represented synthesis in preparation for cell division, a ratio of the order of 20:1 in the mitotic indices should be observed. In fact the number of dividing cells in normal liver is very much

lower than this ratio. Similar comparisons of normal and regenerating liver using ^{32}P to label the DNA show ratios of approximately 1:11 for the specific activities [7, 17, 27]. Fuerst *et al.* [13] have investigated the incorporation of ^{15}N-labelled adenine in this organ and found that ^{15}N derived from adenine is readily incorporated into the adenine and guanine of the DNA in regenerating liver but only about 2 per cent of that specific activity was found in normal liver. It is doubtful whether, in view of the different conditions of the experiments, these differences between the incorporation of labelled adenine and ^{32}P are significant, especially since in the experiments of Fuerst *et al.* [13] the loss of labelled DNA after regeneration is completed, amounts to 1 per cent per day. The incorporation of labelled glycine and formate into liver DNA is similar to that of ^{32}P. Differences in the specific activity of precursors make it difficult to arrive at an accurate estimate but it can be concluded that in addition to some turnover, approximately 1 per cent of the existing DNA is renewed daily in normal adult liver.

The main sources of error in experiments designed to compare the increase in DNA from tracer experiments with the formation of new cells are to be found in evaluating the specific activity of the DNA precursors, the formation of new cells and the contamination of DNA with other labelled compounds. It is however unlikely that these errors are large enough to reduce the values of the ratios by one half, especially since some experiments give the expected ratio (Table II). Euler and Hevesy [11] and Ahlström *et al.* [1] found that both in the liver of young rats and in Jensen sarcoma about twice as much DNA becomes labelled with ^{32}P than is accounted for by the increase in cell numbers. Stevens *et al.* [28] showed that in growing rats the increase in the number of liver cells is 0.71 per cent per day whilst 1.13 per cent (inorganic phosphorus as precursor) or 1.24 per cent (acid-soluble phosphorus as precursor) ^{32}P was incorporated into DNA daily, a factor of 1.6 or 1.75. For similar observations on the intestinal mucosa a factor of 2.7 to 1.8 according to the mode of calculation was obtained. Barnum *et al.* [4] found that in transplanted mouse mammary carcinoma twice as much DNA is formed than can be accounted for by cell formation. Daoust *et al.* [8] determined the rate of cell formation in the lungs of rats as 4.1 per cent daily whilst 8.0 or 10.7 per cent of new DNA-P was formed, a factor of 1.95 or 2.6.

Leblond and his collaborators concluded from these results that completely new sets of DNA are formed in preparation for cell division, but abandoned their hypothesis since later results [14] indicated that only an amount equal to the increase in DNA is formed. Both Nygaard and Rush [19] and Daoust

TABLE II. *New DNA as indicated by labelling divided by the increase in DNA as indicated by cell proliferation.*

Material	DNA labelled / DNA synthesized	References
Jensen sarcoma (rat)	1.5	[11]
Growing liver (rat)	2	[1]
	1.6 –1.75	[28]
	1.0 –1.4[a]	[14]
Regenerating liver (rat)	1.1	[19]
	1.0	[9]
Intestine	1.8 –2.7	[28]
Mammary carcinoma (mouse)	2	[4]
Lung (rat)	1.95–2.6	[8]
E. coli B	1.0[a]	[14]

[a] These figures were derived from the loss of tracer after incorporation, all others from measurements of the rate of incorporation.

et al. [9] found that in regenerating rat liver the formation of DNA as indicated by the amount of ^{32}P incorporated into the DNA is approximately equal to the increase in DNA-P calculated from the growth.

The various experiments of Leblond and his collaborators are of special interest here since they were undertaken under comparable experimental conditions. The data on the incorporation of ^{32}P into the DNA of normal rat liver make it likely that approximately 1 per cent of the DNA is renewed daily and the discrepancies of the findings on growing and regenerating liver can be resolved if this renewal is taken into account. Adding to this figure of 1 per cent the uptake corresponding to a growth rate of 0.71 per cent in growing rats leads to the observed value [28] of DNA synthesis 1.6–1.75 times in excess of cell proliferation. In regenerating liver [9] an increase of 40 per cent in DNA between 18–48 hours after partial hepatectomy is equivalent to a daily increase of approximately 33 per cent and the additional 1 per cent due to normal renewal would only raise the factor from 1 to 1.03, even if it were assumed that regenerating liver functions normally. It is of interest, however, that those observers [9] also estimated the loss of labelled DNA after regeneration and found it to be equivalent to a daily loss of approximately 1 per cent.

The amount of DNA per cell in somatic tissues is twice that of the haploid amount in mature sperm [6, 29], and it must therefore be assumed that DNA synthesis which does not lead to mitosis must either produce hyperploid

amounts or be compensated for by loss of DNA. Cells containing tetraploid amounts of DNA are known to be present in rat liver but their number does not increase in adult rats and the findings that approximately the same proportion of ^{32}P incorporated into DNA is lost as is incorporated indicates a true metabolic turnover. There is, however, some evidence that in some normal tissues nuclei contain up to 10 per cent more DNA than the diploid value [3]. At least 40 per cent of the epithelial cells in the seminal vesicle incorporate labelled adenine at a rate which is approximately one quarter of that found in dividing tissues. The rate of renewal of DNA might therefore be as high as 10 per cent per day, equivalent to a doubling of the total amount in 10 days which in a five month old mouse should have led to many cells carrying at least octoploid amounts of DNA. Such cells were not found and since cell division in this tissue is insufficient to account for the observed DNA synthesis, a loss of DNA equal to synthesis must occur. This loss might be in the form of secretion of DNA itself, of a compound containing components of DNA or loss by exchange of purines. Small Feulgen-positive granules were occasionally observed in the cytoplasm of epithelial cells of the seminal vesicle but their total amount seems to be too small to support a hypothesis of secretion of intact DNA. It is of interest that protein synthesis is unusually heavy in the seminal vesicle [15].

The experiments reviewed here can be explained if it is assumed that renewal of DNA occurs to a varying degree in differentiated cells but that it is connected with their function and not with mitosis. The various results indicate that such metabolic renewal is very low or absent in brain, muscle, adult cartilage and bacteria, but that it occurs at the rate of 0.5–1 per cent daily in liver and kidney and approaches the rates observed in rapidly dividing tissues such as the Jensen rat sarcoma, transplantable mouse mammary carcinoma, lung and intestinal mucosa. Autoradiographs of tissues whose DNA had been labelled with ^{14}C-adenine show no uptake in brain, smooth and skeletal muscle, mature cartilage or epididymis and only weak uptake in liver and kidney. Definite incorporation was observed in the seminal vesicle, as reported in detail in this communication, and in the tracheal epithelium. In mouse prostate glands grown in tissue culture in normal medium [18] 40 per cent of the epithelial cells synthesise DNA during 5 hours. After four days' treatment with 20-methylcholanthrene, although the mitotic index is doubled, the proportion of cells synthesising DNA is unchanged. These findings can be explained on the assumption that normally a considerable proportion of the epithelial cells synthesise DNA without proceeding to division.

There is some evidence that labelled materials can move from the nucleus to the cytoplasm in non-dividing cells. Thus the finding that after injection of ^{32}P the specific activity of the nuclear RNA reaches its maximum before that of the cytoplasmic RNA, has been interpreted to mean that at least some of the cytoplasmic RNA is synthesised by the nucleus. It has been shown by means of autoradiography that some nuclear RNA labelled with ^{32}P appears in the cytoplasm some time after transferring a labelled nucleus to an unlabelled enucleated cell [16]. In tissue cultures of avian squamous epithelium [12] and of mouse prostates [18] unextracted sections show much nuclear and little cytoplasmic labelling 5 hours after applying 8-^{14}C-adenine; after 24 hours there is heavy cytoplasmic labelling but not much increase in the nuclei. Two hours after i.p. injection of [^{35}S-DL]-methionine, the tracheal epithelium of mice shows labelling of the cytoplasm but not of the nuclei and the perinuclear region after 2 hours; after 4 hours 60 per cent of the nuclei are labelled but the perinuclear spaces are still free, after 8 and 16 hours the nuclei become weaker but the perinuclear region positive [20]. These observations indicate that in this tissue proteins are synthesised in nucleus and cytoplasm, and that proteins are secreted from the nucleus into the cytoplasm.

The cytoplasmic labelling of epithelial cells of the seminal vesicle after injection of adenine which remains after treatment with cold perchloric acid is not removed by extraction with hot perchloric acid. Since the treatments have removed RNA or both nucleic acids respectively, the most likely explanation of the findings is that this incorporation represents a different compound, probably protein. The labelling of this cytoplasmic compound is very weak in the seminal vesicles of animals killed 1 day after injection but becomes stronger after 3 and 6 days, indicating a compound which is formed rather slowly. Since the plasma level of labelled adenine is very low 24 hours after injection, it is likely that the ^{14}C utilised is derived from 8-^{14}C-adenine incorporated in the cells but at present it is not known whether the ^{14}C is still bound to adenine.

Reith [23] has recently described compounds of low molecular weight consisting of uridylic acid-aspartic acid isolated from electrophorograms of extracts of mouse liver. It seems possible that similar compounds containing adenine are formed and polymerize slowly, so that under the conditions of the experiments described in this paper they do not appear until some time has elapsed. Billingham *et al.* [5] have found that antigenic properties can be transferred in cell extracts to embryos or very young animals of genetically different strains of mice. From the mode of preparation it is most likely

that desoxyribonucleoproteins are the active components of such extracts and since their activity is destroyed by treatment with either trypsin or desoxyribonuclease it can be assumed that a combination of protein and desoxyribonucleic acid is responsible for their properties.

It is possible that DNA metabolism in non-dividing cells leads to the formation of such compounds or of enzymes containing adenine in their prosthetic groups. This view seems to be supported by the finding that compounds containing ^{14}C derived from labelled adenine or still attached to it are found in fairly high concentration in the cytoplasm of the tissues which show high metabolic activity of DNA, namely intestinal mucosa and seminal vesicle, but not in others. It could be said that the secretion of such compounds into the cytoplasm is one of the functions of the nucleus in some differentiated non-dividing cells.

SUMMARY

Mice were injected with [8-^{14}C]-adenine, killed 1 to 6 days later, organs were fixed in acetic acid-alcohol and formol saline, embedded in paraffin wax and sectioned. After deparaffinising some slides were treated with ribonuclease, deoxyribonuclease, cold perchloric acid or hot perchloric acid and autoradiographs were prepared by the stripping film technique.

Both nuclei and cytoplasm of the epithelial cells of the seminal vesicle of mice killed one day after injection were found to be well labelled in unextracted slides. After extraction with cold perchloric acid or ribonuclease approximately 40 per cent of the nuclei are labelled, their cytoplasm is weakly labelled. No nuclear autoradiograph is shown after extraction with hot perchloric acid, whilst the cytoplasmic autoradiograph remains and probably represents incorporation into protein. Comparison of staining reactions and grain counts indicates that cold perchloric acid is effective in extracting ribonucleic acid. The nuclear autoradiographs observed after cold perchloric acid are therefore due to labelled adenine incorporated in deoxyribonucleic acid.

The seminal vesicle of the mouse has to be regarded as non-dividing tissue and these results therefore indicate DNA synthesis independent of mitosis. The literature on incorporation of tracers in non-dividing tissues is reviewed.

It is suggested that (*a*) synthesis or exchange of DNA takes place in some tissues as a metabolic function unconnected with cell division, and (*b*) that this metabolism is connected with the formation of certain proteins.

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RADIOCHEMICAL INVESTIGATION OF THE UTILISATION OF GLUCOSE BY TISSUE CULTURES

O. SUSCHNY, G. KELLNER, E. BRODA, B. FIGDOR and W. RÜCKER

First Chemical Institute, The University, and Institute of Histology and Embryology, The University, Vienna, Austria

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The catabolism of carbohydrates and other substrates by tissue *in vitro* can be elucidated by incubation in the presence of the radioactive compounds [14]. The utilisation, by different kinds of tissue, of various substrates can be compared, and the presence of the required enzymes verified [12, 15]. In these investigations it has become clear that the metabolism of the tissues depends on experimental conditions. Much more must be known of this influence, before a quantitative comparison of the metabolic pathways in different kinds of tissue can be undertaken, or else false conclusions may quite easily be drawn from a restricted number of data.

It has been pointed out that the behavior of tissue *in vitro*, where it proliferates indefinitely and de-differentiates, must be contrasted with that of tissue *in vivo* where unorganised growth would destroy the organism. If a mechanism operates to control proliferation of cells *in vivo*, its gradual or sudden loss might be reflected in changes in the pattern of metabolism during the early stages of growth *in vitro* [11].

Another factor to be considered is the environment. While in the intact organism the ambient medium changes only within narrow limits, in the medium for tissue culture the concentrations of nutrients and other substances can be varied. The composition of the gas phase and the temperature can also be chosen arbitrarily. Naturally, the rate of metabolism depends on all these factors. Thus, respiration of fibroblasts in different conditions has been followed radiochemically [17], and their influence on the patterns of metabolism has become apparent. For instance, the ratio between aerobic glycolysis and respiration by embryonic chick heart tissue increases with the concentration of the glucose; under nitrogen, glucose is utilised by glycolysis only [5].

It has been the main object of this investigation to study by radiochemical methods the change of respiration and aerobic glycolysis of fibroblasts during the transition to *in vitro* conditions, and their dependence on the concentra-

tion of glucose. Some similar work on HeLa carcinoma tissue will also be reported. The experiments demonstrate the enormous effects on the pattern of metabolism of the stage in transition and of the amount of glucose.

EXPERIMENTAL

Cultivation of tissues.—The chicken fibroblasts were taken from the legs of embryos 10 days old. The carcinoma tissue was derived from a sub-line of HeLa cervix carcinoma [4] kindly supplied by Prof. H. Lettré, Heidelberg. For parallel experiments, pieces of tissue of about equal size were used.

The tissue was grown in roller tubes on a layer of chicken plasma on the glass wall. The medium for the fibroblasts consisted of chick embryo extract, human ascites fluid and Gey's isotonic buffered salt solution (free of glucose) in the ratio 4:40:56. For HeLa, the ascites fluid was replaced by human cord serum. Moderate changes in the composition did not make much difference.

In some cases, monolayer cultures were used [2, 22]. 4–5 days after explantation, the fibroblasts were treated with trypsin at 37°C and pH = 8.5 for 45 min. as described previously [8]. Thereafter, the tissue was centrifuged and washed several times with salt solution. The cells were now suspended in chick embryo extract and counted in a haemocytometer. Finally, the required quantity of cells, with a volume of liquid not exceeding 0.1 ml, was inserted into roller tubes coated with plasma, and left 3–4 hours to settle. Then nutrient medium was added, and the tubes revolved at 14 rph in the drum. After a lag time, in which the cell count decreased to one quarter, the tissue began to grow and to form a monolayer of cells on the plasma surface. Within about 3 days, the original number of cells was reached again. At the end of an experiment, the tissue could be treated with trypsin, and the cells counted again.

At the beginning of each experiment, every tube was supplied with 1 or 2 ml of medium and 0.01–0.1 (in most cases 0.02) μC (micro-curies) of generally labelled radioactive glucose. This had been prepared in Amersham from radioactive CO_2 by photosynthesis, and purified in our laboratories by ion exchange and paper chromatography [16]. The mass of the radioglucose (about 0.1–1.0 μg per tube) was negligible compared with that of the inactive glucose in the medium (500–1000 μg/ml). When necessary, the glucose was determined by the method of Kowarski [10]. Incubation was always carried out in aerobic conditions. The roller tubes were closed by rubber caps, but the supply of oxygen did not decrease appreciably during an experiment.

Separation of the metabolites.—After incubation, the carbon dioxide was recovered from each roller tube. This was connected, by means of an injection needle pushed through the rubber cap and by rubber tubing, to an absorption flask holding 100 ml *n* NaOH. Through a second needle, which reached down into the nutrient medium, air mixed with a known amount of inactive CO_2 was admitted to the roller tube. Before insertion, the longer needle was dipped into silicon grease to prevent foaming. The gas flow was adjusted to 1 bubble/second by suction applied to the absorption flask. Transfer of the radioactive CO_2 was complete within 90 min. The carbonate was precipitated as $BaCO_3$, filtered, washed, dried and stored for measurement.

Now the proteins were precipitated with 2 ml alcohol per ml medium, and removed by centrifugation. A known quantity of lactic acid was added as carrier, the solutions

made up to 100 ml, and shaken for 20 min. with 2 g. Dowex-50 cation exchanger (200–400 mesh). The resin was removed by filtration, and treatment repeated with an equal amount of resin. The basic metabolites, including amino acids, can be recovered from the resin by elution, but neither they nor the proteins were determined in the present investigation.

The filtrate was shaken twice for 20 minutes with 3 g of the weakly basic anion exchanger Dowex-3 to absorb the acid metabolites. Strongly basic resins react with glucose, and therefore cannot be used [13]. Now the resin was shaken 3 times with water to wash out all glucose, and the anions were eluted twice with 100 ml *n* NH_4OH each time. The combined eluates were brought to dryness, and combusted by the wet method of Van Slyke and Folch [18]. The CO_2 obtained was absorbed by *n* NaOH, precipitated as $BaCO_3$, washed, dried and stored for measurement.

The distribution of the radioactivity over the various acids adsorbed on the ion exchanger was examined by paper chromatography with the system propanol: [$NH_3/(NH_4)_2CO_3$ buffer] = 3:1 [6]. The radioactivity of the lactic acid spot far exceeded that of all other spots combined. For the purpose of this investigation, it will be sufficient to consider the total radiocarbon in the acid fraction as being contained in the lactic acid. In a few experiments, not discussed in this paper, the volatile acids were separated from the lactic acid by addition of inactive acetic acid as a carrier, and distillation with dilute sulphuric acid. The radioactivity of the volatile acids was always relatively small. In further experiments, now in progress, the radioactivities of the keto-acids of the citric acid cycle, in various experimental conditions, are measured.

Measurement of radioactivity.—The radioactivity was always measured by filling a gas Geiger counter with CO_2 [1]. In a vacuum line, the CO_2 was set free from the $BaCO_3$ with perchloric acid, dried with $Mg(ClO_4)_2$, and trapped in an U-tube kept in liquid air. After all traces of air had been pumped off, the CO_2 was allowed to evaporate, and its pressure determined with a Hg manometer. Finally, the gas was frozen into the liquid air-cooled glass finger of the Geiger counter, the tap of the counter was closed, and the CO_2 evaporated again. The counter was attached to the filling line by ground joint.

Pressures in the counter were, as a rule, 200–400 mm Hg. Accordingly, operating voltages varied between 3 and 5,5 kilovolts. In most experiments plateaus of 200–400 volts were easily obtained; in case of difficulty, 5 per cent benzene vapour were added from a storage bulb. As no radiation is absorbed in a window or in the sample itself, and geometrical efficiency is essentially 4π, the counting yield is not far below 100 per cent. However, to calculate the amount of radiocarbon in the sample from the count, a correction had to be applied for the yield in recovery. This was done by the formula

$$A = (A_m - B)\, F\, G\, \frac{C}{100}\, \frac{760}{p}\, \frac{22.4}{v}\, \frac{T}{273}.$$

Here A is the total activity of the entire sample, A_m the measured activity, B the background activity, G the weight of the sample (in mg), C the percentage of carbon in the sample, p the pressure of the CO_2 (in mm Hg), v the volume (in ml) of the part of the apparatus connected with the manometer, and T the absolute tempera-

ture. The factor F (1/0.85 for the counting tube here used) allows for the dead volume of the counter.

In practice, the sensitivity of the method, with undiluted radioactive glucose, as obtained by radiophotosynthesis in usual conditions (about 100 μC/mg), is of the order of 10^{-8} mg. The sensitivity can still be improved by using glucose of higher specific activity, by counting for longer periods, or by reducing the background through enhanced shielding and an anti-coincidence circuit. On the other hand, the sensitivity is bound to be less when the radio glucose is diluted by inactive glucose, as in the experiments here described ($\sim 10^{-5}$ mg).

RESULTS

Attention had to paid to the possibility of a production of radioactive CO_2 or lactic acid from glucose by nutrient medium alone. Therefore, blank experiments were carried out in every case by incubating medium, without tissue, in parallel with medium plus tissue. The activities of the CO_2 and of the lactic acid fraction, as found in the blank experiments, were always deducted from the activities obtained in the presence of tissue. The production of radioactive CO_2 in the blanks was negligible, unless (rarely) contamination by microorganisms had happened. More activity was found in the "lactic acid" fraction from the blanks, even without incubation, if the glucose had been sterilised by heating. However, it was shown by paper chromatography, that the activity was due to a mixture of substances, presumably acid decomposition products of glucose. When the glucose had been sterilised in the cold by filtration, the activities of the "lactic acid" in the blanks, without or with incubation, were very low. In the later experiments, only cold sterilisation was used.

In the following tables, the activities found in the lactic acid and in the CO_2 will be expressed as percentages of the activities introduced. The values are, as a rule, mean values from 3 identical parallel runs. The ratio (designated q) of these two percentages gives the ratio of the amounts of glucose carbon converted into lactic acid and into CO_2. While it is suggestive to equate this ratio to the ratio of aerobic glycolysis and respiration, it will be pointed out in the discussion that we have no certainty about the metabolic pathways. Moreover, the rates of the production of the two metabolites may well change during the time intervals considered. Therefore, we prefer to use, at the present stage, the neutral expression "q-value".

In one series the addition of radioglucose was made at different times after explantation (i.e."age" of explants was varied; Table I). The medium was renewed at the start of the experiments, and, except for the presence of

TABLES I—XII

		Radioactive CO_2 produced (%/day)	Radioactive lactic acid produced (%/day)	q
I. *Dependence of metabolism on age of explants (fibroblasts)*				
Time since explantation (days)				
0– 2		0.7	4.8	6.9
2– 3		2.1	7.6	3.6
4– 6		4.3	13	3.0
5– 7		—	—	2.7
15–17		—	—	1.6
II. *Dependence of metabolism on age of explants (monolayers of fibroblasts)*				
Time since explantation (days)	No. of cells (% of orig. explant)			
0– 2	40	0.0033	0.13	39
2– 3	80	0.039	0.76	20
3– 4	76	0.041	0.84	21
10–12	—	0.16	1.20	7.5
III. *Dependence of metabolism on amount of glucose (fibroblasts)*				
Glucose (mg/2 ml)				
2		7.5	8.4	1.1
8		1.1	3.4	3.1
22		0.34	1.2	3.5
42		0.13	0.48	3.7
IV. *Dependence of metabolism on the mass of tissue (fibroblasts)*				
Number of pieces of tissue per roller tube				
30		1.7	2.0	1.2
10		0.31	3.2	10
1		0.06	1.8	30
V. *Metabolism of fibroblasts in salt solution*				
Glucose (mg/2 ml)				
very little		21	3.6	0.17
12		0.51	0.68	1.3
VI. *Metabolism of fibroblasts in dependence on time since change of medium (by difference)*				
Time since explantation (days)	Time since change of medium (hours)			
4	0–4	0.69	5.2	7.5
4	4–24	0.36	4.5	13
5–6	24–48	0.64	8.2	13
6–8	48–96	1.7	3.1	1.8

	Radioactive CO_2 produced (%/day)	Radioactive lactic acid produced (%/day)	q
VII. *Metabolism of fibroblasts in dependence on time since change of medium (determined directly)*			
Time since change of medium (days)			
0	1.2	6.2	5.2
2	1.4	20	14
3	2.2	19	8.6
4	5.9	32	5.4
7	13	26	2.0
VIII. *Metabolism of fibroblasts in dependence on time since change of medium (large amount of tissue)*			
Time since change of medium (days)			
0	6.4	13	2.0
1	7.2	12	1.7
2	15	12	0.8
4	28	9.0	0.3
IX. *Metabolism of fibroblasts in dependence on time since change of medium (large amount of tissue and of glucose)*			
Time since change of medium (days)			
0	1.5	3.2	2.1
1	1.6	4.0	2.5
2	0.94	6.0	5.7
4	1.4	9.5	6.8
X. *Dependence of metabolism on amount of glucose (HeLa)*			
Glucose (mg/2 ml)			
2	1.1	6.7	6.1
8	0.03	1.4	47
XI. *Metabolism of HeLa in salt solution*			
Glucose (mg/2 ml)			
Very little	1.0	7.5	7.5
12	0.06	0.74	12
XII. *Dependence of metabolism of HeLa on time since change of medium*			
Time since change of medium (days)			
(*a*) 0	2.2	7.9	3.6
1	4.1	14	3.4
2	4.1	13	3.2
4	8.9	27	3.0
(*b*) 0	0.20	1.0	5.0
1	0.28	1.2	4.3
4	0.19	1.0	5.3

	Radioactive CO_2 produced (%/day)	Radioactive lactic acid produced (%/day)	q
XIII. *Dependence of metabolism on the mass of tissue (HeLa)*			
Number of pieces of tissue per roller tube			
10	0.69	4.1	6.0
5	0.48	4.0	8.2
1	0.068	0.54	8.0

radioglucose, was identical with the medium used before "radioactive incubation".

Of course, the increasing rate of metabolism partly reflects the growth of the tissue. At the same time, the change in q indicates a qualitative change. Though it will later be shown that q generally decreases with the amount of glucose per unit mass of tissue, the gradual adaptation of the tissue to the new conditions, and the recovery from the shock of explantation may also be factors.

The influence of these factors is very clear in monolayer cultures prepared from cell suspensions, where the decrease in q is very striking (Table II). By counting the cells at the different stages, it was established that the increase in the rate of metabolism is not accounted for by the increase in the number of cells. The increase in metabolism and also the decrease in q must rather be ascribed mainly to the overcoming of the "trypsin shock" and to adaptation.

It might be suspected that cells killed by trypsin contribute to lactic acid production, as given in Table II. In fact, many dead cells were present in the beginning, and were dissolved only gradually. However, experiments indicated that the dead cells do not produce lactic acid from exogenous glucose. Fibroblasts and HeLa were kept at $-10°C$ for 24 hours, and subsequently incubated with radioglucose; no radioactivity was found in either lactic acid or CO_2. It is not excluded, however, that dead cells influence the metabolism of surviving cells.

When tissue is transplanted from one roller tube to another, the value of q increases at first, and returns to the original value after some days only. Apparently, respiration suffers more easily than does glycolysis. Transplantation cannot be carried out in a well defined way, and the increase in q is not well reproducible. In the case of monolayers, where transplantation involves

treatment with trypsin, q is increased to a particularly high value, and approaches the value observed over the first two days of growth, as given in Table II.

To study the influence of the supply of glucose, roller tubes were covered with large amounts of fibroblasts of age 5 days. The tubes were incubated 2 days with nutrient medium containing varying amounts of inactive glucose (Table III). Equal quantities of radioglucose were added to each tube. The salt content of the medium was adjusted to compensate for the osmotic pressure of the glucose.

The activity of the lactic acid was roughly inversely related to the amount of glucose; apparently the quantity of lactic acid formed is not much affected by the concentration of the glucose. In contrast, the activity of the CO_2 decreased more steeply than the specific activity of the glucose. Hence the observed rise in q should be ascribed to a depression of respiration.

The result was even more pronounced when the amount of glucose was kept constant, and the amount of tissue was varied (Table IV). In this series, the tissue had been explanted some weeks earlier, and been transplanted 5 days before the beginning of the experiment. The medium was replaced by a new medium, to which no glucose, except the very small amount of radioglucose, had been added beyond its natural content. Lactic acid production per unit mass was increased to a very high degree as the supply of fuel was improved. Of course, not only the ratio mass of tissue/amount of glucose, but also the ratio mass of tissue/amount of medium varied in this series of experiments.

Extreme conditions are met when the tissue is kept at a minimum level of glucose (i.e. the endogenous glucose of the tissue, and the glucose of the plasma coating). The quantity of radioglucose can be neglected. To exclude the glucose contained in the ordinary nutrient medium, the tissue (from the same batch as in the experiments of Table III) was kept in buffered isotonic salt solution. The medium was poured off, and the tissue rinsed with salt solution before the start of the experiment (Table V).

It appears that, in spite of the unsuitability of the system for growth or prolonged survival, small amounts of glucose are used very effectively. For comparison, 12 mg glucose was added to the saline in parallel experiments. In this case, production of CO_2 from glucose was still about as good as if a genuine nutrient medium had been present (see Table III).

In further experiments, the metabolism was measured as a function of time while the nutrient medium was not changed. The medium contained only the natural glucose of its biogenic constituents (about 2 mg at the start).

Explants of age 4 days were used and the radioglucose was added together with the fresh medium. Roller tubes were incubated for different times, and radioactive CO_2 and lactic acid determined. By computing the differences, the production of the two substances during the intervals of time was obtained (Table VI).

More precise results could be expected from experiments, where radioglucose of high specific activity, dissolved in a very small volume of salt solution, was added to roller tubes at various times after the last change of medium. Here the production of radioactive CO_2 and lactic acid, during the different intervals, could be determined directly (Table VII). The tissue had been explanted 3 days before the beginning of the experiment, and incubation lasted 8 hours.

The maxima of q in Tables VI and VII point to the existence of two opposing influences. In order to find how these factors depend on the relative "fuel supply", this was decreased, in another set of experiments, by packing the tubes with large amounts of tissue (Table VIII). The duration of radioactive incubation was one day. It is seen that here only the trend known from Table III is apparent. The situation corresponds to that at the later stages of the experiments of Tables VI and VII.

To some more roller tubes, also packed with large amounts of tissue, 6 mg of inactive glucose were fed along with the radioglucose (Table IX). In this way the concentration of the glucose was kept practically on the same level for each experiment. The figures for the production of radioactive CO_2 and lactic acid show that the amount of glucose is large enough so that only a small part is utilised; no fuel shortage is felt. The gradual increase observed in q may, as in the first stages of the experiments of Tables VI and VII, perhaps be ascribed to the gradual deterioration of the system (medium and/or tissue).

With HeLa carcinoma, a series of experiments in analogy to Table III was made (Table X). The tissue had been transplanted 3 days before the beginning of the experiments. Radioactive incubation lasted 2 days. Curiously, both the production of radioactive CO_2 and lactic acid dropped to very low levels on addition of 22 or 42 mg of glucose.

Of great interest is the high value of q found when tissue (from the same batch) was kept in salt solution for 2 days (Table XI; compare Table V).

Metabolism seems to change less with time elapsed since change of medium in HeLa than in fibroblasts. In Table XII*a* and *b*, two sets of results are reproduced in complete analogy to Tables VIII and IX respectively. Duration of radioactive incubation was 1 day.

In some respects, the results of Table XII are similar to the corresponding

results with fibroblasts. However, the cancer tissue does not show the precipitous drop of q as the supply of fuel decreases (Table XII*a*). Nor does the q-value in HeLa markedly decrease with increasing mass of tissue (Table III). This result again contrasts with the result obtained with fibroblasts (Table IV).

DISCUSSION

In general, the production of radioactive lactic acid and CO_2 cannot be referred to the number of cells; except in the experiments of Table II, the numbers of cells were not known. In any case, our experience as well as that of other authors [3, 24, 25, 26] indicates no proportionality between the numbers of the cells in the tissue and the rate of metabolism. Thus, only the ratios between the amounts of lactic acid and CO_2, produced from radioactive glucose, will be considered (q-values).

The q-values are based on the net production of radioactive lactic acid and CO_2 during rather long time intervals. Very probably in some of the experiments at first lactic acid was formed from the glucose, and part of the acid oxidised to CO_2 later. Though in a number of experiments we have found comparatively little production of CO_2 from radioactive lactic acid by fibroblasts in presence of glucose, the lactic acid may well be strongly attacked as the supply of glucose runs out [11, 23]. In any case, it is questionable to what extent the utilisation of endogenous lactic acid is reflected in the utilisation of lactic acid from the medium.

In principle, incorporation of radioactive carbon from CO_2 must also be expected. However, in practice no significant activity of the carbonic acids of the tissue was found after incubation of fibroblasts or HeLa with radioactive bicarbonate for 24 hours.

The lactic acid is probably largely formed by the Embden-Meyerhof-Parnas pathway, and the CO_2 by the citric acid cycle [21, 23]. However, in both respects the hexose monophosphate shunt may have contributed. Yet from the point of view of the total energy supply the nature of the pathways is of secondary importance. Whatever the mechanisms, the present experiments lead to conclusions as to the extent to which the tissue, in various conditions, derives its energy from "burning" or from "splitting" glucose.

Obviously the value of q is very sensitive to changes in conditions. The lability of aerobic glycolysis as well as the ease, with which respiration is damaged, have been stressed by Warburg [19, 20]. In particular, many of our experiments, mostly not reported here, show that q increases sharply (and

not well reproducibly) as tissue is subjected to influences expected to be unfavourable. Values of 30 and more are obtained easily by subjecting tissue, e.g. fibroblasts, to sub-optimal conditions. Such tissue is usually recognised as damaged by observation with the microscope. The gradual increase of q on keeping the tissue in one and the same medium, with replenishment of glucose only, for several days, may also be interpreted as being due to deterioration of conditions.

On the other hand, tissue has a certain capacity to adapt itself to new conditions. Thus, q decreases with time after explantation (Tables I and II). It appears as if the fibroblasts overcame the shock of explantation and adjusted themselves to life *in vitro*. Similar but less striking results have been obtained after transplantation of either fibroblasts or HeLa between roller tubes.

The value of q also depends on the amount of metabolic fuel per unit mass (Tables III and IV). There is a tendency to waste fuel by not completely burning it, if it is abundant. Conversely, if the supply is very restricted, the fuel is fully utilised (Table V).

The increase of q with improving fuel supply is also noted with HeLa (Table X). But in contrast to the position with fibroblasts, reduction of the fuel supply even to the lowest level will not depress q below a certain fairly high value (Tables XI and XII*a*). Increasing the mass of HeLa tissue also fails to produce a significant effect (Table XIII).

The absolute numerical values of q, as derived from fibroblasts on the one hand and from HeLa on the other hand, should not be compared. Little is known about the dependence of q on the origin and the developmental stage of the tissue at the time of explantation, and on the animal species. Yet some differences between the fibroblasts and the tumour tissue here examined appear significant. While the q-value of fibroblasts can be depressed to values so low that far more glucose is burnt than split, no such depression has so far been observed by us with HeLa.

On the other hand, though high q-values can be achieved for both fibroblasts and HeLa, it must not be concluded that respiration is equally dispensable for both kinds of tissue. Radiochemical tests, e.g., have shown that HeLa survives treatment with high concentrations of cyanide, while fibroblasts do not [7, 9]. These experiences throw light on the qualitatively different roles played by respiration and by glycolysis in normal and in cancer tissue.

SUMMARY

The aerobic production of carbon dioxide and of lactic acid from radioactive glucose by chicken fibroblasts and by HeLa tumour *in vitro* has been investigated. The ratio of glucose carbon converted into lactic acid and into carbon dioxide, respectively, has been called q-value.

Generally, the q-values of fibroblasts are low in conditions favourable for survival. After explantation or transplantation, the q-values are high, and go down only as the tissue adapts itself to new conditions.

However, scarcity of the metabolic fuel glucose also tends to decrease the value of q, and very low q-values are observed when the fibroblasts are kept starving in isotonic salt solution.

With HeLa, no conditions have been found where respiration predominates over glycolysis. Though for both HeLa and fibroblasts conditions can be adjusted so that far more glucose is glycolysed than oxidised, respiration seems to play a role in fibroblasts, which is qualitatively different from that in HeLa.

In view of the strong dependence of q on experimental conditions, these must be standardised strictly, if different kinds of tissue are to be compared.

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THE EFFECTS OF CHELATING AGENTS ON CELL DIVISION

D. DAVIDSON

Department of Botany, University of Oxford, Oxford, England

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It has been suggested that calcium and magnesium ions play an important role in maintaining chromosome structure [1–5, 7, 8]. The removal of these ions from the cell sap would reveal how important they were; for example, chromosome breaks might appear.

Plants deficient for certain ions can be produced by growing them in nutrient solutions in which those ions are not present, but it is a long process [7, 8]. Rapid inactivation of metallic ions in solutions can be achieved, however, by using chelating agents.

Ethylene-diaminotetra-acetic acid (EDTA), and quinaldinic acid are chelating agents. When added to solutions containing metallic ions they form chelate-metal complexes. Though soluble, these complexes are almost unionized. The cations are not removed from solution, but are rendered ineffective; the condition simulates a cationic deficiency. EDTA shows a marked affinity for calcium ions. Quinaldinic acid, on the other hand, is more effective with ions of heavier metals, such as copper and manganese.

The effects of quinaldinic acid and EDTA on plants could be due to the rapid removal of metallic ions from the cell sap. The effects have been studied in root-tips and in shoots. The results confirm and extend those of McDonald and Kaufmann [6], who also used plant cells.

MATERIAL AND METHODS

Bulbs of *Allium cepa* were grown over distilled water. Treatments were begun when the roots were 1–2 cm long and they were made with 0.001 and 0.0001 *M* solutions of EDTA, 0.002 and 0.0002 *M* quinaldinic acid and 0.01 *M* $CaCl_2$, $MgCl_2$ and NaCl. Treatments lasted up to 72 hours and the roots were then returned to water. The roots were fixed 4–144 hours from the beginning of treatment in acetic-alcohol (1/3) and prepared as Feulgen squashes.

Cut shoots of *Tradescantia paludosa* (Oak Ridge Clone V) were dipped into 0.01 *M*, 0.001 *M* or 0.0001 *M* solutions of EDTA. After one week, treated shoots were kept in distilled water. Pollen mother cells and pollen grains were prepared as aceto-carmine squashes, 1–14 days from the beginning of treatment.

The experiments were made in temperature cabinets at 20–21°C. Double glass distilled water was used for the preparation of all solutions and in control treatments; the ion content of such water is extremely low.

RESULTS

Roots.—The effects of treatment with EDTA or quinaldinic acid for up to 24 hours were similar. They were: sticky chromatid bridges at anaphase, swollen chromosomes at metaphase and mitotic inhibition. Chromosome breakage was not found.

No other effects were seen after 0.0002 *M* quinaldinic acid and the 0.002 *M* solution stopped all root growth. Treatments with quinaldinic acid were therefore not continued. They had shown however that the action of EDTA is probably not to be attributed solely to the inactivation of calcium ions, since the two compounds are so similar in their effects.

After 48 hours in 0.001 *M* solutions of EDTA, arrested metaphases showing a typical C-mitosis were found, and restitution nuclei were also seen. After a further 24 hours in the EDTA solution arrested metaphases in diploid cells continued to occur, and for the first time tetraploid metaphases appeared. In three tetraploid cells, diplochromosomes were present indicating that the centromeres of sister-chromatids had not separated at the previous mitosis.

When the onions were returned to distilled water after 72 hours' treatment, no new changes were observed and no roots were fixed after 3 days' recovery. Chromosome breakage was not found during either the period of treatment or recovery (cf. [6]).

Other onions were kept in 0.001 *M* solutions of EDTA for 48 hours and then transferred to 0.001 *M* solutions of $CaCl_2$, $MgCl_2$ or NaCl for 6 hours. Roots fixed at the end of this 6-hour period or after 18 hours in water showed no change in the effects of the treatment with EDTA. The failure of free calcium and magnesium ions to induce a return to normal mitosis was unexpected, but it is now understandable in view of the results of treatments

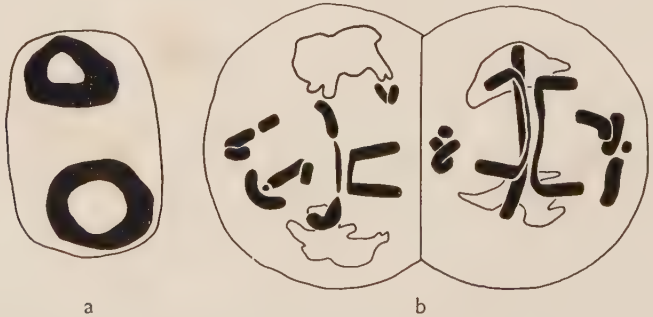

Fig. 1.—*Tradescantia paludosa* pollen mother cells: (*a*) TI, 0.01 *M* EDTA, 3 days. Ring nuclei. × 330. (*b*) AII, 0.001 *M* EDTA, 6 days plus 5 days' recovery. Bridges and fragments. × 345.

with solutions of calcium and magnesium salts. $CaCl_2$ and $MgCl_2$ solutions by themselves produce mitotic abnormalities similar to those produced by EDTA [6].

Shoots.—The first effect on pollen mother cells resembled that seen in roots. Sticky bridges appeared, at both first and second anaphase; laggard chromosomes and chromatids were also present and gave rise to micronuclei.

Other effects were also observed. In some pollen mother cells from shoots kept in 0.01 *M* EDTA for 3 days, and in pollen grains from shoots kept in 0.001 *M* EDTA for 4–7 days, nuclear reconstitution had been upset. The telophase nuclei formed were not compact, but instead, they took the form of large rings (Fig. 1 *a*). In pollen mother cells from the same shoots as the pollen grains wall-formation had been suppressed, the four products of meiosis were not separated and bi- or quadri-nucleate cells were formed.

In shoots kept in EDTA solutions for six or seven days, some pollen mother cells at anaphase contained fragments, and in other cells the bridges present were obviously not due to stickiness. These were the first signs that chromosome breakage could occur after treatment with EDTA, but the frequency of affected cells was always 5 per cent or less; thus on these results EDTA cannot be considered an efficient agent for the induction of chromosome breakage.

Extensive breakage and reunion did not occur until the shoots were returned, after six or seven days' treatment, to distilled water. The breakage then found was in second, not in first, divisions (Fig. 1 *b*) and, like other abnormalities, was sporadic. Not all anthers in a bud showed effects, but within an anther all the cells might be affected. Affected cells occurred up to 15 days after the beginning of treatment.

DISCUSSION

In undertaking these experiments it was hoped that EDTA would reveal *in vivo* the specificity for certain cations that it shows *in vitro*, but these hopes were not realised. With the possible exception of the inhibition of wall formation, all the effects induced by EDTA also occur after treatment with quinaldinic acid and 8-oxyquinoline [9]. In particular, chromosome breakage appears after treatment with EDTA and 8-oxyquinoline. The breakage may have been caused by the inactivation of calcium ions, but we cannot argue that this is so from the results of the experiments described here, nor have we any evidence to support the view that ionic bonds occur in chromosomes. The wide range of effects of chelating agents indicates that many cations become

combined in chelate-metal complexes as a consequence of which probably many reactions are affected and the general metabolism of the cell is upset [6]. A further complication is the fact that affected cells and tissues appear sporadically, i.e. effects are localized, indicating variation in cell sensitivity.

It is not yet possible to attribute an effect found after treatment with a chelating agent to the inactivation of certain ions. This will occur only when a chelating agent is known which shows *in vivo* a specificity equal to that obtained *in vitro* under precisely defined conditions.

SUMMARY

Root-tips of *Allium cepa* and flowering shoots of *Tradescantia paludosa* were treated with solutions of chelating agents (EDTA and quinaldinic acid) and $CaCl_2$, $MgCl_2$ and NaCl. Various abnormalities were found at meiosis and mitosis. Chromosome breakage occurred but only in shoots and only after a long delay. The chelates do not appear to be as specific *in vivo* as they are *in vitro*. The effects found after treatment cannot therefore be attributed to the inactivation of particular cations.

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STUDIES ON THE ADAPTATION OF YEAST TO COPPER

XIX. EFFECT OF COPPER ON CYTOCHROME COMPONENTS OF YEAST

T. MINAGAWA[1]

Biological Institute, Faculty of Science, Kobe University, Himeji Branch, Himeji, Japan

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According to unpublished studies by the author and his collaborators, copper-cultured yeast differs from normal yeast in the aerobic respiration. It has been reported that the presence of a low copper concentration results in an increase in cytochrome *a* and a more or less weakening of the absorption bands of cytochromes *b* and *c* [5, 24]. The report presented here deals with the effect of a higher concentration of copper on cytochrome components of yeast.

MATERIALS AND METHODS

Unless otherwise specified strain B (Kyoto) of *Saccharomyces ellipsoideus* was used, cultured in PGY medium (peptone 2 g, sodium glutamate 2 g, yeast extract 2 g, $MgSO_4 \cdot 7H_2O$ 1 g, $(NH_4)_2SO_4$ 1 g, KH_2PO_4 3 g, $CaCl_2$ 0.25 g, cane sugar 50 g, and distilled water one l), and the copper-resistant strain called R_{1b}, which was obtained by training the former in the PGY medium poisoned with 1 m*M* of $CuSO_4$. The cells were cultured at 30°C in the Roux bottle, 200 ml of the liquid medium being contained to form a layer of 7.5 mm thickness. Compared with the parent strain growing in the normal medium, R_{1b} began growing after a little longer lag and grew somewhat slower in the copper-medium. However, each of the strains reached the stationary phase in 20 to 24 hours after inoculation. Copper-cultured cells became brown-colored after about the middle of the logarithmic phase and were deep brown in the stationary phase.

Cells were washed two or three times, spread on cover glass as thick paste, dithionite was added, and the absorption spectra were observed using a microspectroscope made by Tokyo Optical Co. Light absorption curves of extracts were determined with Shimadzu's spectrophotometer. Spectroscopic observations were carried out at room temperature, unless otherwise notified.

RESULTS

Spectroscopic observation of cells.—The deep color of R_{1b} cells interfered with spectroscopic observation. This coloration is assumed to be due chiefly to

[1] Present address: Department of Zoology, Syracuse University, Syracuse, New York, U.S.A.

copper sulfide [1, 13], and cells are easily decolorized by addition of KCN or $FeCl_3$. Hence, for the observation of R_{1b} cells, one of these agents was added to the cells, followed by repeated washing with water or disodium ethylendiaminetetraacetate solution. This procedure was not considered to affect the absorption spectra of the cytochromes, because (1) the spectra of parent cells were not changed by the same treatment, and (2), as described later, the spectra of the R_{1b} cells thus treated were the same as those of the particle preparation isolated from untreated R_{1b} cells and observed without the treatment.

The parent strain cultured in the standing liquid medium for 40 hours showed the band of cytochrome *c* at 550 mμ and the weak band of cytochrome *b* at 565 mμ, cytochrome *a* not being detectable. R_{1b} cultured in 1 m*M* copper medium for 40 hours lacked the bands of both cytochromes *a* and *c*, only the absorption at 565 mμ being distinct. Weak absorption band of cytochrome *c*, however, became observable with the R_{1b} strain when the culture was three or four days old. By the addition of pyridine, the 565 mμ band of R_{1b} was shifted to a shorter wavelength and intensified; the same result was observed with isolated particle from parent cells. With the parent cells a strong continuous absorption ranging from 550 to 560 mμ was shown.

When parent cells were heated at 80°C for 20 minutes, followed by the decolorizing treatment, the cytochrome *c* band remained as such, the cytochrome *b* band disappeared, and a weak diffuse band around 580 mμ was newly formed, as reported by Ephrussi *et al.* [6] for baker's yeast. Cytochrome *c* has been reported to be resistant to heat exposure [6, 11], and Ephrussi *et al.* have inferred from their experiment that the new band at 580 mμ may be due to the heat denatured protein and the prosthetic group of thermolabile cytochrome *a* [6]. The inference may be applied to the present case of parent cells. In the case of R_{1b} cells, however, neither the extinction of the 565 mμ band nor the formation of the 580 mμ band was caused by the heat treatment. Observed at the temperature of liquid air, the 565 mμ band of R_{1b} cells, either heat exposed or not, shifted to a wavelength that was shorter by a few mμ, as did cytochrome *b*. At this temperature R_{1b} sometimes showed a diffuse band around 580 mμ, which was not detectable at room temperature. In the baker's yeast observed by Ephrussi *et al.* [6], a distinct absorption near 560 mμ arose when cells were exposed to heat and observed at liquid air temperature. But similar results were not observed with the parent strain B (Kyoto) used in the present studies.

When normal cells were harvested after 10 hours of culture in normal medium or when R_{1b} cells were harvested after 10 hours of culture in 1 m*M*

copper medium, the cells absorbed only between 555 and 560 mμ, the band being diffuse and thermolabile. This band may represent cytochrome b_1 [6] or b_2 [23]. It could not be determined whether the cells at the stationary phase had this band.

Cytochromes *a*, *b* and *c* were observable when cells were cultured for 40 hours on media solidified by 3 per cent of agar. In this case, the band of cytochrome *c* was the strongest, *b* the next and *a* the weakest when using the parent strain. The strongest in R_{1b}, however, was the 565 mμ band, which was not diminished by heat exposure, just as in the case of liquid culture.

The absorption spectra of the two strains were compared after they were cultured in liquid media containing various concentrations of $CuSO_4$. The growth of the parent strain was not significantly inhibited below 0.05 m*M* of copper, but was somewhat inhibited at 0.1 m*M*. In the spectra of this strain grown in 0.025 m*M* copper-medium, the absorption of cytochrome *c* was almost as intense as that of cytochrome *b*. Grown in 0.05 m*M* copper medium, however, it showed feeble cytochrome *c* band and the thermostable 565 mμ band, and grown in 0.1 m*M* copper medium the latter band only. The R_{1b} cultured in the medium without copper addition represented the same absorption bands as the parent strain cultured in the normal medium. And when it was cultured in copper media, the spectra were the same as the parent strain grown in media containing the same concentrations of copper.

The results showed that a component absorbing at 565 mμ was formed in cells of the both strains when the medium contained 0.05 m*M* or more of copper. The component was heat stable, although its absorption band falls on that of cytochrome *b*. The formation of that component was accompanied by the disappearance of cytochrome *c*. Such a disturbance in the cytochrome formation was caused by copper in the resistant strain, as in the sensitive one, whereas the former was not inhibited in its physiological activities such as growth and respiration by the copper concentration used.

In the next place, a synthetic medium supplemented with vitamins was used. The resistant strain was obtained by training the parent strain in this medium to which 0.1 m*M* of $CuSO_4$ was added, as copper was more toxic in it than in PGY medium [12]. The parent strain and the resistant strain were cultured without and with addition of copper, respectively. Cells were harvested at the stationary phase. The spectral absorption of the cells was very weak at room temperature. But the observations at the temperature of liquid air provided the results similar to those described for cells cultured with PGY medium.

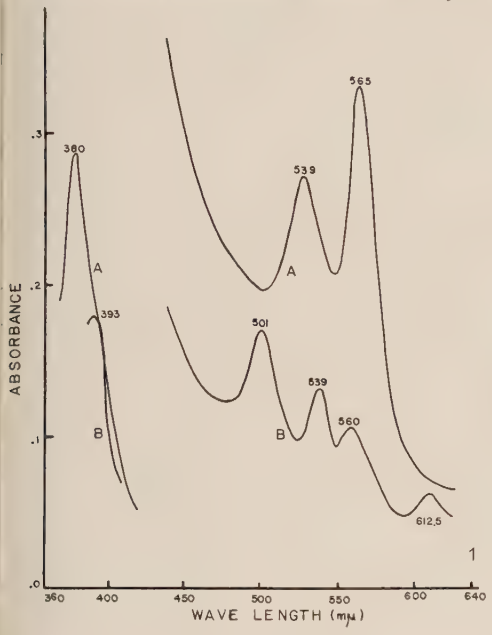
Fractionation of disrupted cells.—Washed cells were ground with two volumes of sea sand, and then extracted with the same volume of 0.2 *M* phosphate buffer at pH 8. The extract was centrifuged at 5,000 r.p.m. for 15 minutes, and the precipitate was re-extracted with the buffer and centrifuged. The two supernatants were joined together and centrifuged at 25,000 r.p.m. (approx. 57,000 *g*) for 20 minutes. Starting from parent cells the supernatant thus obtained was yellow and almost transparent, and the pellet consisted of an upper pinkish-grey layer and a lower white one. From R_{1b} cells, the supernatant was turbid and brown, and the pellet was dark brown carrying a thin white layer at the bottom. When the supernatant from R_{1b} was centrifuged at 40,000 r.p.m. (approx. 110,000 *g*) for 20 minutes it separated into three fractions, almost clear reddish-brown supernatant, white light precipitate and the dark brown compact pellet. The second could easily be removed by pipette, leaving the third.

Each of the precipitates was washed with and suspended in phosphate buffer, and observed spectroscopically in the presence of dithionite. Absorption bands could be observed without decolorizing the colored particles. However, the observation was easier when they were decolorized by the KCN treatment.

Cytochrome *c* only was observed in the yellow supernatant of parent cells, and a weak band of cytochrome *c* and a strong band of *b* were observed in the pinkish-grey fraction thereof. The dark brown pellet of R_{1b} had the thermostable 565 mμ band, while the white precipitate and the reddish-brown supernatant by centrifugation at 40,000 r.p.m. had no absorption band in the visible range. The absorption band shown by the dark brown particulate fraction of R_{1b} coincided with that by cytochrome *b* of the colored particulate fraction of parent cells, both at room temperature and at liquid air temperature. The absorption band of the former fraction was shifted by pyridine just like that of the latter. But the former was resistant to the heat exposure, and was neither oxidizable in the presence of ferricyanate nor by aeration. In this respect, the substance absorbing 565 mμ in the copper-cultured cells was not identified as cytochrome *b*.

Identification of the 565 mμ substance of R_{1b}.—The dark brown particulate fraction of R_{1b} was extracted with sodium cholate after the method used to extract cytochrome *b* from heart muscle [18]. But only a small amount of light absorbing substance was extracted after prolonged incubation at 30°C. Repeated extraction with 0.2 *M* NaOH from washed cells, or extraction with 80 per cent acetone from cells which had been treated with 0.2 NaOH were both effective, the second method more so than the first. Precipitation oc-

curred when the extract was made pH 5 by adding dilute HCl. The precipitate was dissolved in a small amount of 0.2 *M* NaOH and reprecipitated by dilute HCl. The precipitate thus obtained was dissolved in 1 *M* NaOH, and 8 volumes of acetone were added. The precipitate formed was removed by centrifugation. The acetone extract was made pH 4.5 by dilute HCl, and green precipitate formed was taken off by centrifugation or filtration through folded



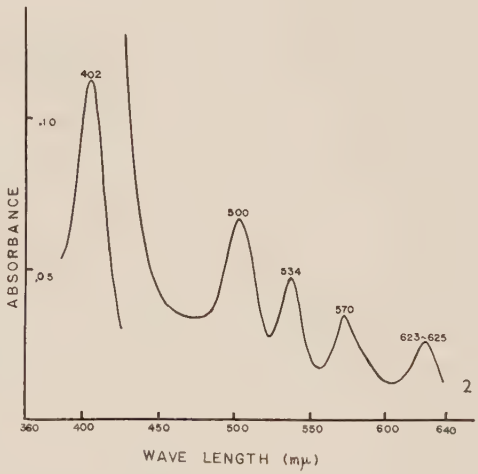


Fig. 1.—Spectra of the pigment from R_{1b} cells (*A*) dissolved in 0.1 *M* NaOH, and (*B*) after reduction by sodium amalgam. Soret region spectra determined with 5 times dilution.

Fig. 2.—Spectra of the porphyrin methylester of the pigment in chloroform. Soret region spectra determined with 5 times dilution.

paper. When the clear pink solution thus obtained was chromatographed on talc column, which had been washed with dilute HCl, a pink pigment remained at the upper end of the column. The column was washed successively with 0.1 *M* HCl, water and methanol, until the wash of each agent became negative of the copper ion by diethyldithiocarbamate. The pigment was then eluted with the mixture of methanol and concentrated NH_4OH (20 : 1, v/v). The pink eluate was dried under reduced pressure.

The absorption curve of the pigment dissolved in 0.1 *M* NaOH is shown in Fig. 1*A*. The NaOH solution of the pigment was neutralized with HCl, and pyridine or ferricyanate was added. But the absorption did not change.

The pigment was ashed with concentrated H_2SO_4 and H_2O_2 in order to test for metals. Copper was detected positive with diethyldithiocarbamate and dithizon, while iron was negative by $\alpha\alpha'$-dipyridil and *o*-phenanthroline in the presence of hydroquinone. Stern *et al.* [19] have reported that, when octasubstituted porphyrin forms a complex with copper, the characteristic four maxima of its absorption curve in the visible region are replaced by the two maxima, higher α-absorption and lower β-absorption, and the red fluorescence of porphyrin disappears. The above-mentioned pink pigment had no red fluorescence. It had blue fluorescence at first, but this became weaker and weaker as the purification proceeded.

Following the procedure of removing copper from turacin (copper uroporphyrin) [14], sodium amalgam was added to the pigment dissolved in 0.1 *M* NaOH, shaken, and bubbled by air stream. After this procedure the absorption spectra as shown in Fig. 1 *B* was obtained. The absorption became weaker by amalgam treatment. The conspicuous two maxima were replaced by four new maxima. The solution fluoresced red strongly, and this fluorescing substance moved easily into ether-acetic acid. Hence the pigment deprived of copper is an ether soluble porphyrin, and the thermostable 565 mμ substance probably is a complex of porphyrin and copper.

The porphyrin obtained was purified using a talc column, and was then esterified after Takagi *et al.* [21]. The absorption curve of the methylester in chloroform is shown in Fig. 2. Thus the porphyrin was identified as coproporphyrin I or III, since the absorption maxima observed were in good agreement with those reported for them [4, 7, 11].

DISCUSSION

Oxygen uptake of cells was measured by the usual manometric method in the presence of 0.005 *M* glucose. Q_{O_2} of cells of the parent strain in the normal medium and of R_{1b} in the copper medium were 17.8 ± 1.97 and 26.0 ± 2.95, respectively, when they were 10 to 14 hours of age, and were 47.0 ± 1.0 and 24.3 ± 2.79, respectively, when they were 40 to 48 hours of age. Proceeding from the logarithmic to the stationary phase, the parent strain in the normal medium increased its oxygen uptake considerably, while R_{1b} in the copper medium did not. This may be consistent with the result reported above, that cytochrome *c* increased as the culture aged in the normal medium, but it was hardly synthesized in the copper medium. It has been reported that the oxygen consumption is roughly proportional to the cytochrome *c* content in tissues of rat [2, 16], and that the respiration of yeast

increases, attended by an increase in cytochrome *c*, when anaerobically grown cells are allowed to be in aerobic conditions [19].

There remains, however, the problem of why R_{1b}, which has no significant amount of cytochromes, is able to take up oxygen even more than the normal cells in the logarithmic phase. This is being studied. The copper-trained strain grows in the copper medium nearly as rapidly as in the normal medium, in spite of the fact that the synthesis of cytochrome *c* is strongly inhibited by copper.

It has been reported that intact cells [9, 10] and autolysing cells, as well as press juice, of yeast [14] can synthesize coproporphyrin. When copper is present in the cell, coproporphyrin will form the copper complex [cf. 3], which will probably be bound by particles in the cell. Keilin [8] has shown that copper uroporphyrin (turacin) plays no role in oxidation and reduction in the cell, and Taylor [22] obtained no evidence to show that the synthetic copper mesoporphyrin has a catalytic action in relation to oxidation and reduction. Copper coproporphyrin does not seem to participate in cellular respiration, since its light absorption was not altered by aeration or by the addition of ferricyanate and dithionite.

Complex formation by coproporphyrin may reduce the amount of free copper in the cell, thus reducing the injury by copper. However, the copper in the complex with coproporphyrin seems to constitute only a very small fraction of the total bound copper contained in the copper-cultured R_{1b} cells at the stationary phase of growth. Much of intracellular copper seems to be detoxified by being precipitated as sulfide [1, 13].

The absorption at 565 mμ observed with copper-cultured cells, or with the particle preparation from them, was much intensified and was shifted to a shorter wavelength by the addition of pyridine. Since the absorption of copper-coproporphyrin is not affected by pyridine, some substance(s) other than copper-coproporphyrin must contribute, together with this, to the absorption at 565 mμ of the copper-cultured cells. The synthesis of cytochrome *b* does not seem to be inhibited by copper so seriously as cytochrome *c*, since, when either the parent strain or R_{1b} was grown in the presence of 0.025 m*M* of copper, the band of cytochrome *b* was not weaker than that of *b* in either cells of the control culture, while that of cytochrome *c* was conspicuously weaker than the control. And even with cells growing in the presence of 1 m*M* of copper, the band corresponding to cytochrome b_1 or b_2 was observable if they were still in the logarithmic phase.

It may, therefore, be concluded that the copper ion added to yeast culture inhibits the formation of cytochrome *c*, and that the 565 mμ band of the cop-

per-cultured yeast is attributable to the copper-coproporphyrin complex and a heme, which seems to be a cytochrome *b* component at least under certain conditions.

SUMMARY

1. The formation of cytochrome *c* by yeast cells during the logarithmic and early stationary phase was inhibited by copper ion. Cells cultured in the presence of copper ion possessed a thermostable absorption band at 565 mμ.

2. The substance chiefly responsible for the latter absorption was identified as the copper complex of coproporphyrin.

3. A heme, probably cytochrome *b*, also contributes to the 565 mμ band partly.

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ELECTRON MICROSCOPY OF THE FALLOPIAN TUBE EPITHELIUM OF RABBITS IN OESTRUS

O. NILSSON

Department of Histology and Laboratory of Ultrastructure Research of the Department of Anatomy, Karolinska Institutet, Stockholm, Sweden

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Electron microscope studies of the epithelium of the rabbit Fallopian tube under different hormonal influences were carried out by Borell, Nilsson, Wersäll, and Westman [5]. Their investigation demonstrated some of the cyclical changes of the epithelial cells.

The most characteristic feature in the epithelium of the rabbit oviduct during oestrus is the production of secretory granules in the secretory cells. The present investigation was undertaken in order to study the process of secretion in this epithelium.

MATERIALS AND METHODS

Six animals in oestrus were used. Laparotomy was carried out under nembutal anaesthesia and a glass cannula was inserted into the tube. A small quantity of a 1 per cent buffered osmium tetroxide solution [16, 23] was injected through the cannula. The distal part of isthmus was then removed, placed in the fixing solution, and cut into small pieces. The pieces were fixed for three hours at room temperature, embedded in *n*-butylmethacrylate [13], and polymerized at + 45°C.

The sectioning was performed with an ultramicrotome designed by Sjöstrand [26] and the sections were examined in an RCA EMU 2c electron microscope.

RESULTS

The epithelium of the rabbit Fallopian tube is of the single, columnar type and rests on a basement membrane. Two types of epithelial cells have been found, viz. secretory cells and ciliated cells. Occasionally leucocytes are also encountered in the epithelium (Fig. 1).

Secretory cells

During oestrus the secretory cells have a very characteristic appearance due to the immense production of secretory granules. The upper surfaces

of these cells facing the tubal lumen bulge markedly beyond the free surfaces of the ciliated cells and the balloon-like bulges are filled with secretory granules (Figs. 1, 2, 3).

The cell membrane.—The cell membrane shows numerous filiform projections on that part which constitutes the upper surface membrane.

The filiform projections have a diameter of about 800 Å and a length of 0.6–0.8 μ. The secretory cells possess as a rule only filiform projections. But in a few cases a structure similar to a cilium with its basal corpuscle was found (Fig. 3).

The intercellular space varies in appearance in its course from the upper cell surface to the basement membrane (Fig. 1). Close to the upper surface for a distance of about 0.6 μ the cell borders run parallel and have a slightly wavy course. The adjacent cytoplasm has in this part a high opacity corresponding to the terminal bars. For the next few microns the borders are still parallel but are not straight and form mutual cell identations. Then the main part of the intercellular space follows, which is mostly of a very irregular shape. The cell membranes no longer run parallel here but make long and tiny projections into the intercellular space. This irregularity is especially marked in the basal part.

The intercellular space is wider between two secretory cells than between a secretory cell and a ciliated cell, and a secretory cell is provided with longer projections into the intercellular space than is a ciliated cell.

The indentations of the cell membranes form in sections structures of varying profiles. But all these are characterized by two bordering membranes and an interior of cytoplasmic appearance (Fig. 11).

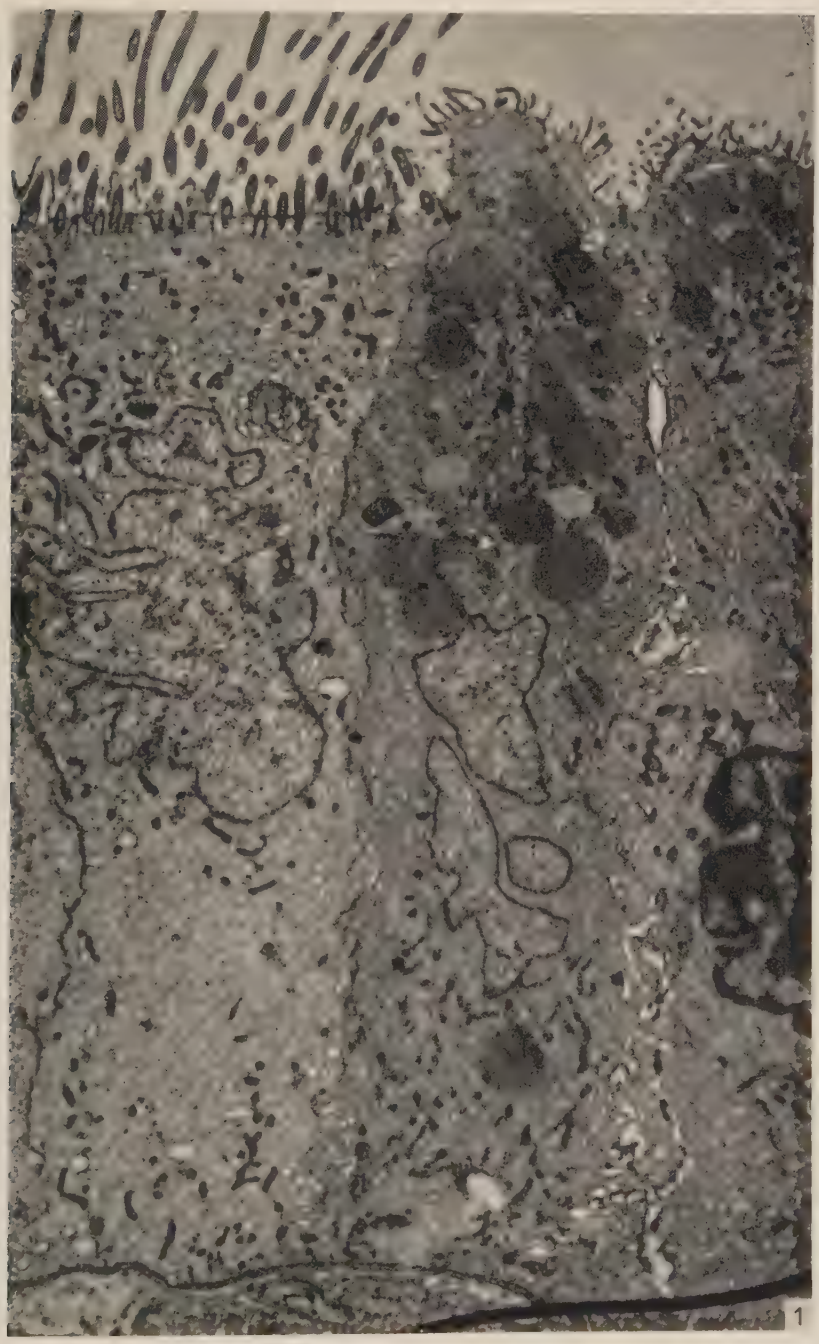
The cytoplasm.—The ground substance of the cytoplasm is homogeneous and only slightly osmiophilic. Several formed constituents are found comprising, *inter alia*, Golgi zone, mitochondria, opaque particles, α-cytomembranes, secretory granules, secretion spaces, vesicles, and fine fibrils.

The Golgi zone, mostly located above the nucleus, has the same general appearance found in other cell types and shows parallel membranes, circular or ellipsoid structures, and vacuoles.

The mitochondria are distributed throughout the cytoplasm and show an inner membrane structure.

Opaque particles are scattered in the cytoplasm. They appear in aggregates

Fig. 1.—Survey picture of epithelium of rabbit Fallopian tube with ciliated cells *(left)* and secretory cells *(right)*. Most of the secretory granules are found in the upper part of the secretory cells. A lymphocyte is partly visible to the right in the middle of the epithelium. The basement membrane and connective tissue are seen in the lower part of the picture. × 8500.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of 5–10 and in connection with cytomembranes (Figs. 5, 6, 7, 10). The secretory cell contains more opaque particles than does the ciliated cell.

A cytomembrane system with attached opaque particles (α-cytomembranes, Sjöstrand [27]) can be recognized throughout the cell (see in particular Figs. 6, 7, 9). The membranes enclose irregular spaces which are filled with a homogeneous substance, slightly more opaque than the ground substance. No opaque particles can be seen in the spaces but they are found attached to the outer side of the enclosing membrane. The α-cytomembrane system is very irregular but sometimes the membranes show a parallel distribution (Fig. 10).

The α-cytomembranes appear much more abundant in the secretory cells than in the ciliated cells (Figs. 5, 6). As they are rather osmiophilic, the cytoplasm of the secretory cells appears dense as compared with that of the ciliated cells (Figs. 1, 2).

A membrane also follows the nuclear membrane very closely, so that the whole nucleus will appear enclosed by an α-cytomembrane (Fig. 6). The small granules are attached to that side of the membrane which faces away from the nucleus. This membrane sometimes bulges into the cytoplasm.

The secretory granules appear as rounded bodies with a diameter of 0.5 - 1.5 μ. They are found throughout the cell but appear in greater numbers in its upper part. The secretory granules consist of an osmiophilic, homogeneous substance which shows small areas with a higher density (Fig. 8). Sometimes homogeneous granules of a low density can be found. The granules seem to be bordered by a membrane (Fig. 10), but often the membrane is only partly visible or apparently lacking (Figs. 3, 8).

Special spaces, here called secretion spaces, can be found in all parts of the cell even immediately under the surface membrane. The secretion spaces have the form of the secretory granules. They are bordered by a membrane and are filled with a substance slightly denser than the ground substance of the cytoplasm. Part of the secretion space is occupied by an irregularly outlined structure showing the density of a secretory granule (Figs. 3, 9, 10). Sometimes the regular figure of a secretion space is broken by an outpocketing into the cytoplasm.

Vesicles can appear anywhere in the cytoplasm as spaces with a diam-

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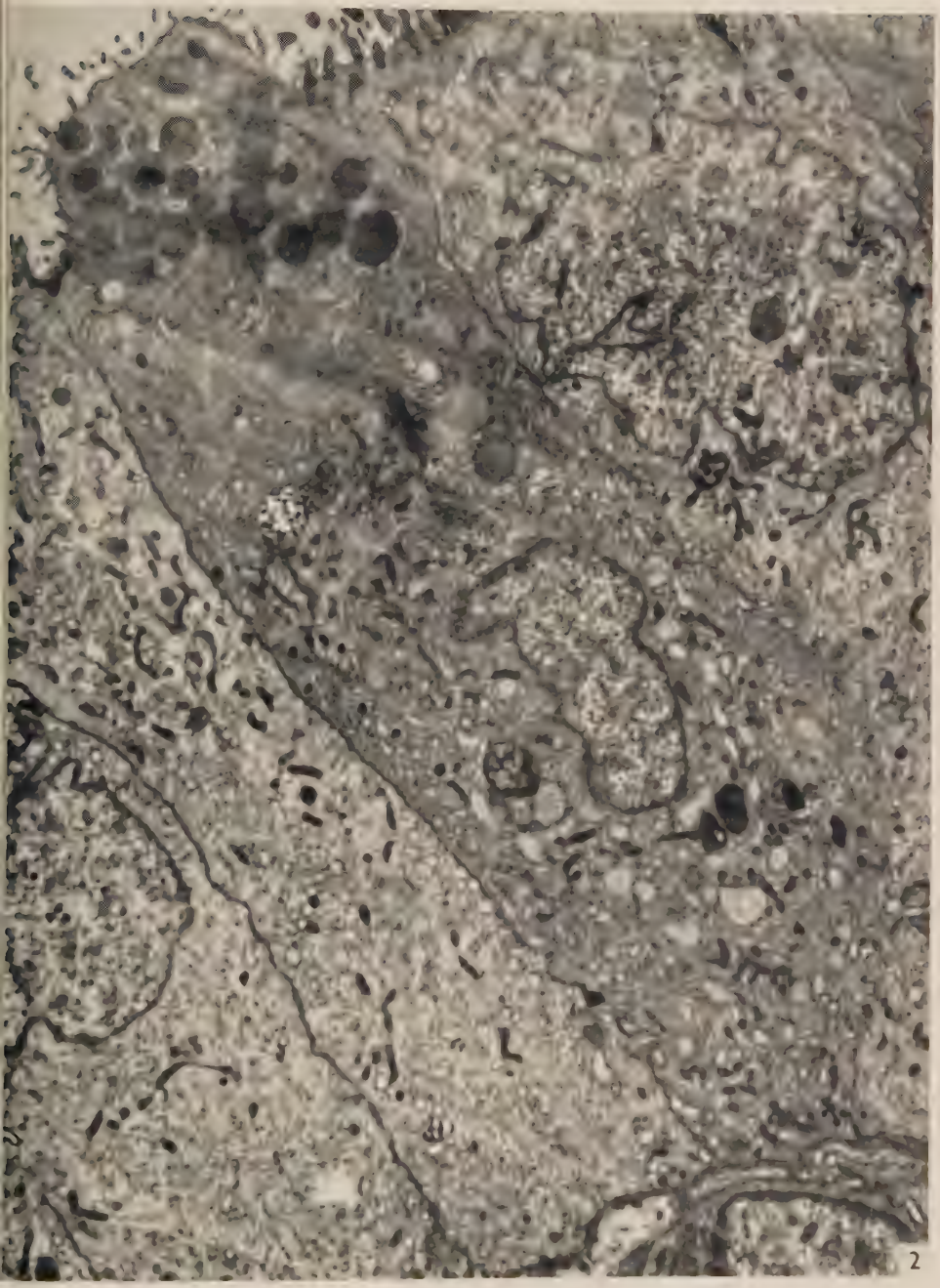
Fig. 2.—Survey picture of epithelium of rabbit Fallopian tube with a secretory cell surrounded by ciliated cells. Juxta-nuclear bodies are lying beside the lower left part of the nuclei. A vesicle containing an irregular structure above and to the left of the nucleus of the secretory cell. The nucleus of the right, ciliated cell shows two nucleoli with a rounded homogeneous appearance. The basement membrane and connective tissue in the lower right part of the picture. × 9000.



eter of about 1 μ, bordered by a distinct membrane. They do not contain any ground substance but the enclosed space shows in sections irregulary scattered granules and filaments of different size (Fig. 2).

Very fine fibrils running in bundles can be followed in the cytoplasm for a length of about 0.6 μ (Fig. 7).

The nucleus.—The nucleus is very irregularly outlined. It is enclosed by an α-cytomembrane which follows all its indentations and which also can be seen forming outpocketings into the cytoplasm. The smooth side of the α-cytomembrane faces the nuclear membrane.

The nuclear membrane consists of two osmiophilic layers separated by a less osmiophilic layer (Fig. 6). In tangential sections several ring-shaped structures can be seen along the nuclear border. They are about 500 Å wide and have a less opaque center.

The nucleoplasm contains a varying amount of small granules. Nucleoli are encountered in the form of irregular structures of a dense material.

Ciliated cells

The intracellular structure of the ciliated cells is somewhat the same during oestrus as during the other phases of the sexual cycle.

The cell membrane.—This has the same general appearance as described for the cell membrane of secretory cells. Filiform processes are seen all over the cell surfaces between the cilia.

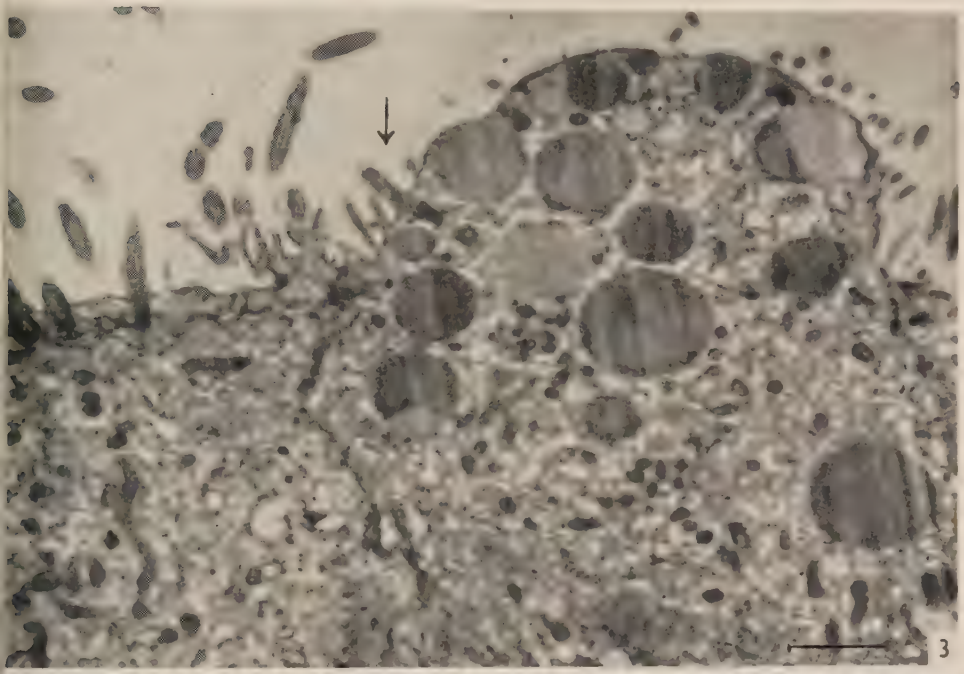
The cilia are covered by a membrane divided into three layers, two of them opaque on either side of a less opaque layer. Their interior contains a ring of 9 peripheral double filaments and two central filaments. Below the cell surface the filaments take part in the formation of the basal corpuscle.

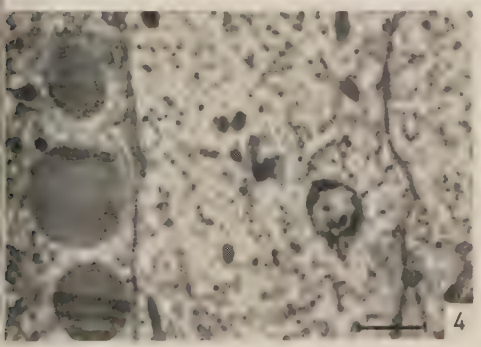
The intercellular space is narrow and regular between two neighbouring ciliated cells but between a ciliated and a secretory cell this space shows greater irregularity (Fig. 1). The cytoplasm adjacent to the spaces has a high opacity in the region of the terminal bars.

The cytoplasm.—The mitochondria and the Golgi zone (Fig. 11) have the same appearance as has been found in the secretory cells.

Opaque particles are found both attached to the cytomembranes and scattered in the cytoplasm. In the cytoplasm of the ciliated cells they are also lying in clusters but occur in a smaller number than in the cytoplasm of the secretory cells (Figs. 5, 6).

The α-cytomembrane system is very poorly developed in the ciliated cells compared with the secretory cells. An α-cytomembrane also envelops the nucleus of the ciliated cells and follows all its indentations (Fig. 12).





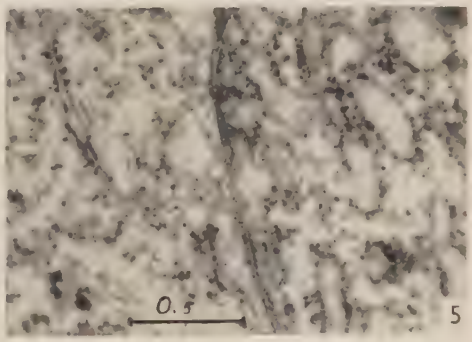


Fig. 3.—Upper part of tubal epithelium. Secretory cell *(right)*, its free surface bulging beyond the free surface of a ciliated cell *(left)*. The secretory cell contains secretory granules and secretion spaces. The arrow points at a single cilium on its free surface. × 13,000.

Fig. 4.—Part of secretory cell with secretory granules *(left)* and ciliated cells *(right)*. A juxtanuclear body is seen in the central ciliated cell. × 9500.

Fig. 5.—Part of ciliated cell *(left)* and secretory cell *(right)* showing the difference in amount of α-cytomembranes and free opaque particles in the cytoplasm of these two cell types. × 31,500.

Vesicles, about 1 μ in diameter, with irregularly scattered granules and filaments are also encountered in the cytoplasm of ciliated cells.

Ring-like structures are often found in sections of the cytoplasm. Besides those connected to the Golgi zone and those formed by indentations of the cell membranes (Fig. 11) two types of different magnitude and of different organization have been found. (1) Ring-like structures with a diameter of about 0.3 μ and bordered by a membrane with two osmiophilic layers are encountered centrally in the cell. Their interior is partly filled with small ring-shaped bodies about 300 Å thick. (2) Small circular bodies about 300 Å in diameter with a distinct bordering membrane are found in irregular clusters. These groups are mostly situated in the lower part of the cell.

Juxta-nuclear bodies (Figs. 2, 4) are found both in the secretory and the ciliated cells. They are rounded structures with a diameter of about 1 μ, and lie close to one side of the nucleus or in an indentation of the nuclear membrane. The juxta-nuclear bodies are bounded by a rather thick membrane and contain in their ground substance small amounts of an irregularly scattered substance. This substance shows a higher osmiophilia than does the material inside the nucleus.

The nucleus.—The nuclear envelope of the ciliated cells shows the same appearances as does that of the secretory cell. The nucleoplasm of ciliated cells possesses besides the granular aggregates that form the nucleoli, also oval, homogeneous bodies with a diameter of 0.5–0.7 μ (Figs. 2, 12).

Lymphocytes

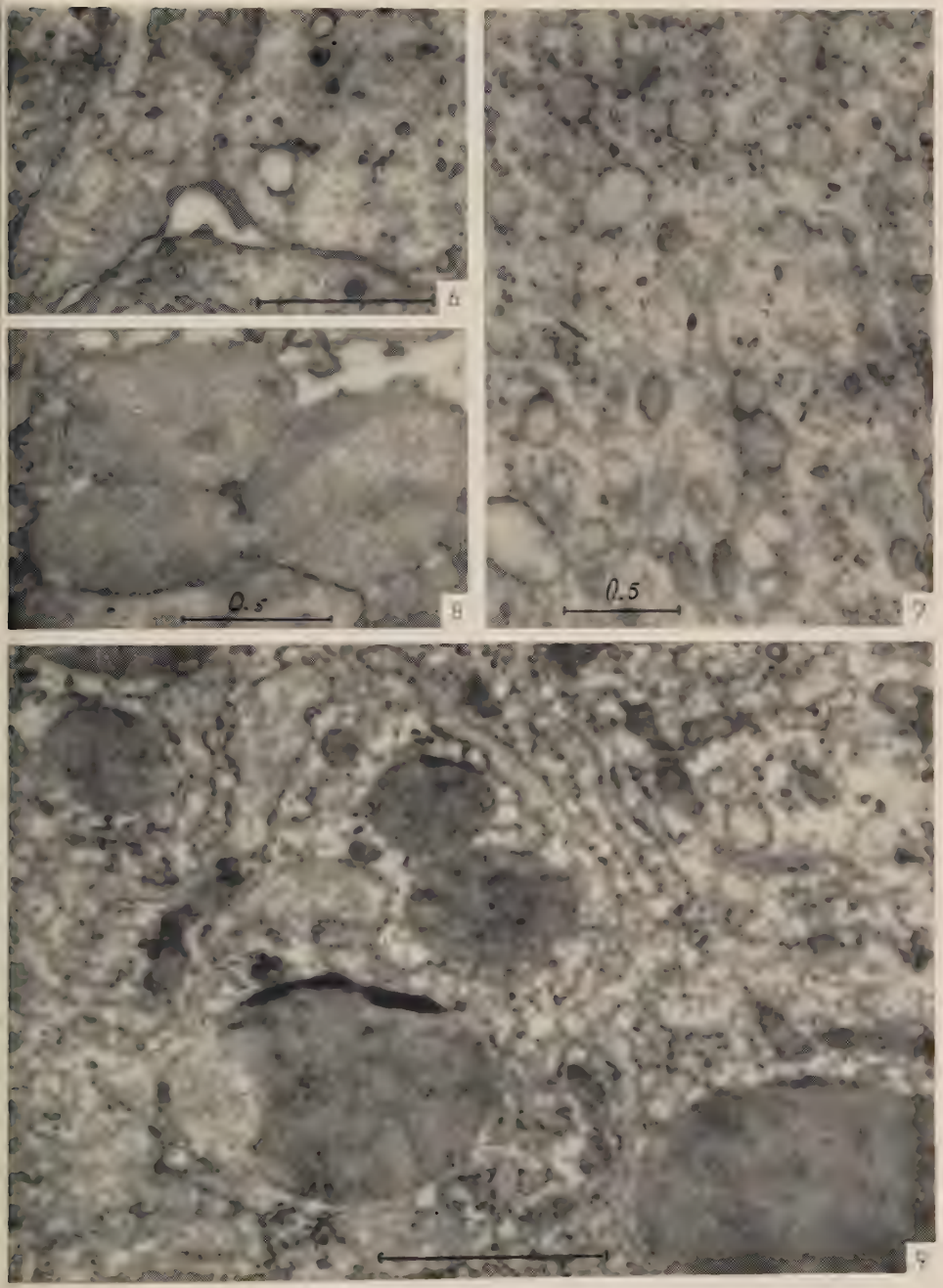
Lymphocytes are sometimes encountered in the basal parts of the epithelium. They have a rounded cell-form with a diameter of about 6 μ and contain in the centre a large, dense nucleus. The α-cytomembranes of the cytoplasm occur sparsely.

Fig. 6.—Part of secretory cells *(left)* and ciliated cell *(right)* showing the difference in amount of α-cytomembranes and free opaque particles in the cytoplasm of these two cell types. The clusters of the opaque particles are clearly visible in the ciliated cell. A part of the nucleus in the upper left corner. The nuclear membrane is double layered and the nucleus is surrounded by an α-cytomembrane. × 24,000.

Fig. 7.—Part of a secretory cell. Profiles of the α-cytomembrane system, clusters of opaque particles, mitochondriae, and fibrillar structures are seen. × 33,000.

Fig. 8.—Secretory granules of rabbit Fallopian tube. Border membranes are partly visible. × 40,000.

Fig. 9.—Part of cytoplasm of secretory cell showing mitochondriae, α-cytomembranes, opaque particles, secretory granules and in the lower left part a secretion space. × 31,000.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DISCUSSION

The secretory cells of the Fallopian tube, from rabbits in oestrus, possess several structural qualities which differentiate them from the ciliated cells. The secretory cells have, in particular, an extensively developed system of granulated cytomembranes and their cytoplasm contains more scattered opaque granules than does the cytoplasm of the ciliated cells. They contain a lot of secretory granules and they have not been found to show a ciliated upper surface as do the ciliated cells.

In this paper, the granulated membranes are called α-cytomembranes, according to Sjöstrand [27]. Similar structures have also been named submicroscopic filaments [1], lamellar structure [8], endoplasmic reticulum [21], ergastoplasm [30], etc.

The α-cytomembrane system of the secretory cells of the rabbit oviduct forms irregular spaces and not a regular system of parallel membranes as for instance in the exocrine cells of the pancreas. But sometimes parallel membranes are found with a concentric distribution around some cell organell (cf. [20]). No definite conclusion can be drawn from this investigation as to what extent the α-cytomembranes of these cells form a tubular system or mainly consist of isolated vesicles.

The opaque particles [28] or the dense particulate component [17], presumably containing ribonucleic acid [20], occur both attached to the cytomembranes and as free granules, mostly lying in clusters, in the cytoplasm. The secretory cells show a large number of opaque particles. This is not only a consequence of all their cytomembranes but is due also to the occurrence of a greater number of free particles in the cytoplasm of the secretory cells than in the ciliated cells.

The great number of α-cytomembranes and opaque particles in the cytoplasm of secretory cells makes the assumption probable that these membranes take part in the production of secretory granules [3, 19, 28, 30]. In the rabbit oviduct the granules are probably synthesized in membrane-bounded spaces in the cytoplasm. These so-called secretion spaces contain

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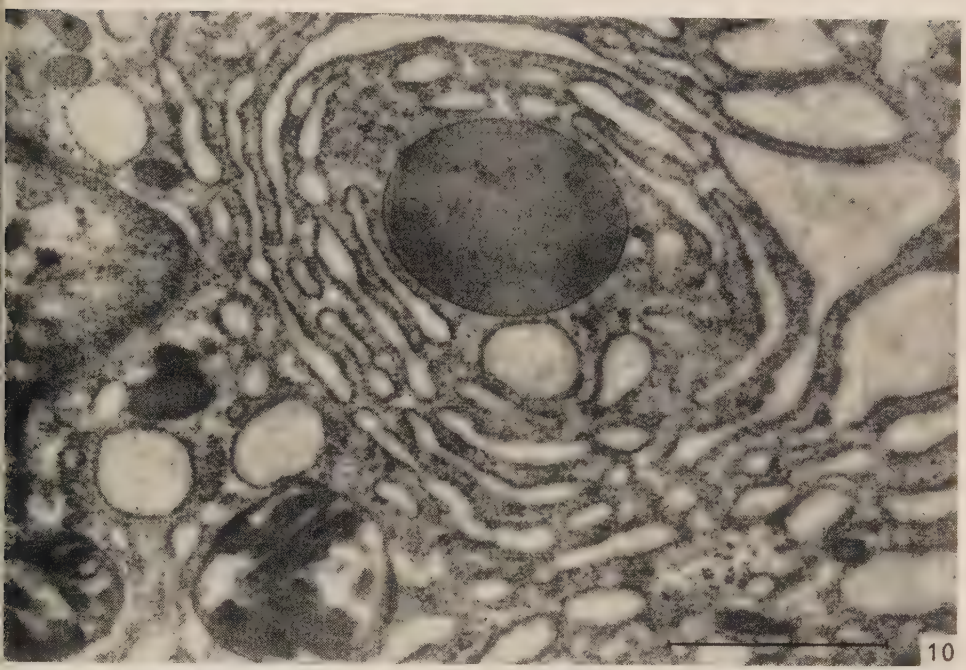
Fig. 10.—Part of cytoplasm of secretory cell. A secretory granule with a distinct membrane lies in the cytoplasm surrounded by α-cytomembranes. Two secretion spaces in the lower left part of the picture. Part of the nucleus is seen to the left. × 30.000.

Fig. 11.—Part of cytoplasm of ciliated cells. The cell border runs in the upper part of the picture. Golgi apparatus in the middle and lower right part. × 14,500.

Fig. 12.—Part of nucleus of ciliated cell showing the different kinds of nucleoli. The nucleus is surrounded by an α-cytomembrane. × 15,500.



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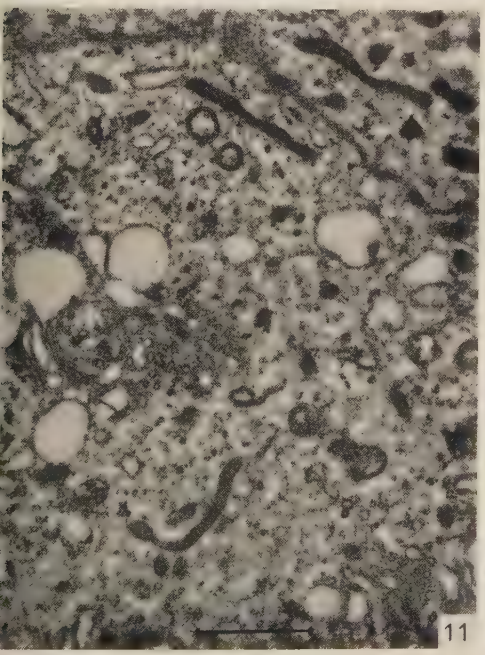

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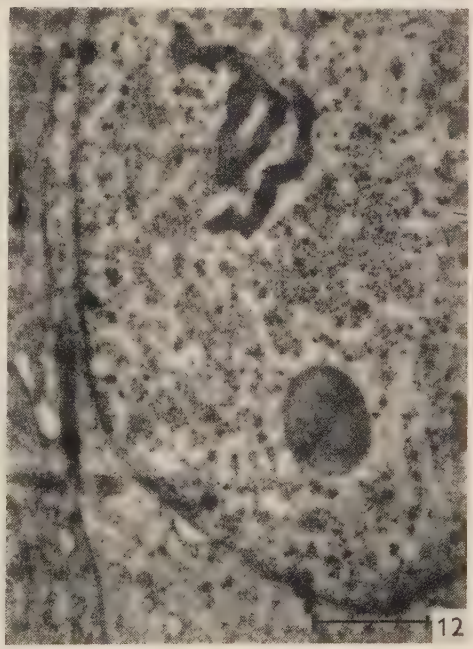

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a substance which exhibits the same osmiophilia as the secretory granules. The substance is found in varying degrees and forms up to the size and form of secretory granules [5]. Somewhat the same picture has been found in other cells producing secretory granules [3, 15, 30, 33]. The origin of these structures is not known but the assumption has been made that they could be equivalent to a dilated cisterna of the α-cytomembrane system [30, 33].

In the secretory cells of the rabbit oviduct the profiles of the α-cytomembrane system vary in size and form up to that of secretion spaces. The present investigation, however, does not permit any conclusions to be drawn as to the identity of the two structures.

The Golgi apparatus has been found to be a site for condensation of secretory products in the exocrine cells of the pancreas [28]. A relationship between the structures of the Golgi zone and the secretion spaces of the Fallopian tube epithelium has, however, not yet been demonstrated.

The secretory granules sometimes show a well developed border membrane but this can mostly only be partly discerned. This membrane probably derives from the membrane of the secretion space and during the development of the granule it may be altered in composition or dissolved. No internal structure has been found in the secretory granules. They seem to have the same general appearance as the zymogen granules of the pancreas [28].

Structures with a ring-like appearance in the sections and partly filled with small ring-shaped profiles have also been found in the epithelium of the mouse uterus [14], the epithelium of the rat oviduct [15], the ciliated cells of the mouse trachea [25] and the absorbing cells of the mouse jejunum [34]. The significance of these vesicles is unknown.

The juxta-nuclear bodies are found in both secretory and ciliated cells. They contain material with a higher degree of osmiophilia than the substance of the nucleus. This makes it improbable that they are a part of the nucleus. The juxta-nuclear bodies are rather large but are seldom encountered, which might imply that only one body occurs in each cell. Their localization, form and size is reminiscent of the centrioles as described in light microscope studies. However, in the electron microscope investigations the centrioles have been found to show the appearance of the basal corpuscle of a kinocilium [2, 6, 22], or to be a rounded body with internal granules and membranes [34]. Thus the relationship of the juxta-nuclear bodies to the centrioles is uncertain.

An α-cytomembrane has been found to enclose the nucleus of many different cells [18, 28, 29] and the relationship of this membrane to the nuclear membrane has been discussed. It has been supposed that the nuclear enve-

lope should be continuous with the membranes of the endoplasmic reticulum [18, 29]. In this study no contact between the enveloping α-cytomembrane and the nuclear membrane of the secretory and ciliated cells has been found.

According to some authors the secretory and ciliated cells of the rabbit oviduct have the ability to transform from the one type to the other [7, 11, 12, 32]. In this investigation no convincing signs of any transformation have been found. This is in accordance with the results from other investigations [5, 10]. The occurrence in a few cases of a single cilium on a secretory cell is regarded by the author not as a proof for transitional forms of cells, but as an interesting cytological event. No cell containing secretory granules and an ordinary ciliated upper surface has been found.

The occurrence of single kinocilia on cells of other characters than the cells of ciliated epithelia is moreover not an unusual finding. Thus structures with the pattern of kinocilia have been found for instance on the sensory cells of the frog olfactory epithelium [4], in the retina [9], on the cells of the proximal convolute of the mouse kidney [24] and on the hair cells of the cristae ampullares in the guinea pig [31].

Lymphocytes are not an unusual finding in epithelial membranes. They have been shown in particular in respiratory epithelium [25] and in the epithelium of the mouse jejunum [34].

SUMMARY

The epithelium of the Fallopian tube of rabbits in oestrus has been found to contain secretory cells and ciliated cells. Occasionally lymphocytes are encountered.

The secretory cells show balloon-like bulges which are filled with secretory granules. The secretory granules are homogeneous and sometimes possess a limiting membrane. They seem to be synthetized in membrane-bounded spaces, here called secretion spaces, which can be found anywhere in the cytoplasm. No proof of a relationship between the secretion spaces and the structures of the Golgi zone or the system of granulated cytomembranes (α-cytomembranes) can be given. α-Cytomembranes form a well-developed system of vesicles. Opaque particles occur in clusters in the cytoplasm in a higher degree in the secretory cells than in the ciliated cells.

The ciliated cells possess cilia which show the usual structural pattern. The cytoplasm of the ciliated cells contains only a few α-cytomembranes as compared with the cytoplasm of the secretory cells. An α-cytomembrane surrounds the nucleus of both ciliated and secretory cells.

The assumption that secretory cells can transform into ciliated cells or vice versa cannot be confirmed although in a few cases a single cilium has been found on a secretory cell.

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THE ELECTRON MICROSCOPY OF THE DEVELOPING HUMAN RENAL GLOMERULUS[1]

S. M. KURTZ

Electron Microscopy Laboratory, Departments of Anatomy and Pathology, University of Alabama Medical Center, Birmingham, Alabama, U.S.A.

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The interest in the structure of the mammalian kidney has received new impetus in recent years with the development of improved techniques for studying tissue sections with the electron microscope. While the tubular cells of the kidney are shown to be not far different from other body cells in basic morphology, electron microscope studies of the glomerulus reveal a wealth of structural details that hardly could have been anticipated. New nomenclature, such as "podocyte", "pedicel", "lamina densa", and "lamina fenestrata" [6] was added to the literature describing the glomerulus. Since the same structures with minor variations were reported by many investigators for a variety of mammalian forms, little dispute arose among investigators in the field as to the presence or interpretation of these structures. However, the general organizational pattern of the glomerulus is still a source of sharp disagreement, in spite of the excellence of electron microscope studies of this structure in the past few years.

The present work was undertaken with the anticipation that electron microscopic studies of the developing human renal glomerulus would yield valuable information concerning the complicated structure of the mature glomerulus, particularly since one of the principal characterizations of the widely accepted concept of "mesangium" [22] is based upon the invagination of the renal corpuscle to form a glomerular stalk or mesentary. In addition to gaining insight into this problem, the observations reported in this work reveal other details which are interesting from the standpoint of histogenesis and developmental anatomy. These latter observations will receive the major share of attention in this report.

MATERIALS AND METHODS

Since our ultimate aim was the interpretation of the human glomerulus, we elected to use the human fetus for the present study, in spite of the obvious disad-

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vantage of intervention of *post mortem* changes, which is impossible to circumvent when working with this material. We were fortunate enough to secure some specimens within one hour following death, and remarkably good preservation of gross and minute structures was obtained (see plates). The tissue was prepared in the following fashion.

(1) After stripping the capsule, 1 to 2 mm slices were removed tangential to the surface of the kidney, since glomerular development begins in this portion. The area including and adjacent to the junctions of lobes was utilized, since previous observations with the light microscope revealed more primitive glomeruli in these regions.

(2) The slices of tissue were immersed with continuous agitation for 1–2 hours in 1 per cent osmic acid buffered to pH 7.4 with veronal acetate [14]. The remainder of the technical procedure was carried out as described by Farquhar [4], and observations were made with the Philips EM 100B microscope at 60 KV.

General Appearance

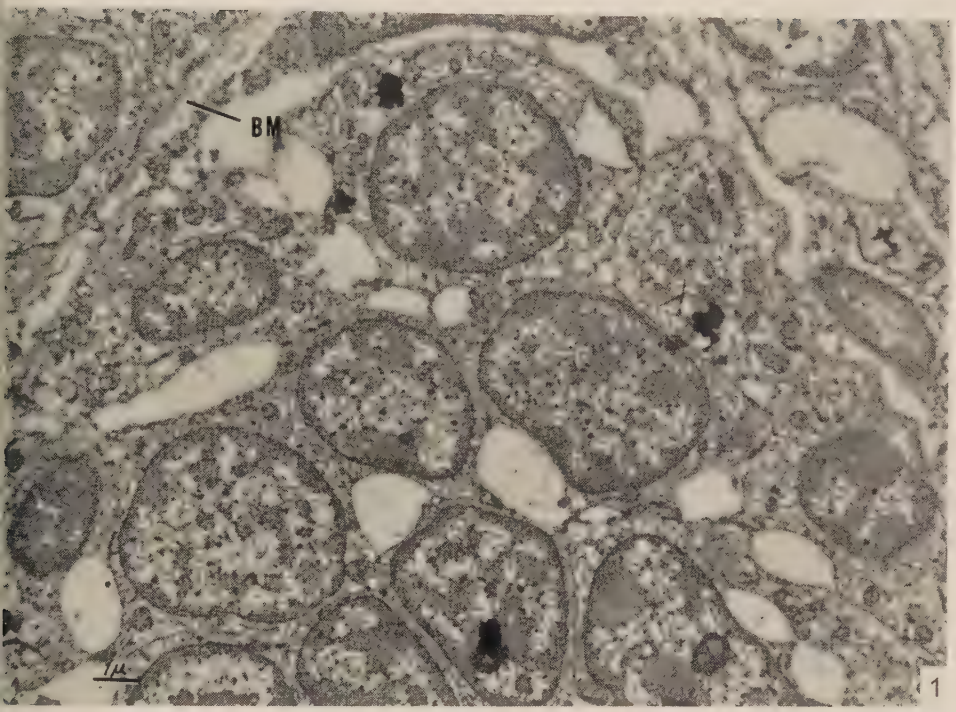
As was anticipated, the bulk of tissue observed in the electron microscope was not comparable in quality to animal tissues fixed instantly while in the living state. However, some specimens, including one fixed at least four hours *post mortem*, exhibited remarkably good preservation of fine structure. One specimen fixed, reportedly, 45 minutes *post mortem* looked as poor as any fixed after the longest lapse of time. Hence, it was not possible to predict the quality of preservation of tissue elements merely upon the lapse of time between death and fixation. It was necessary not only to sample each specimen, but to sample from many tissue blocks and several areas of the same block.

Even the poorest of preparations yielded some worthwhile information, for the tissue elements, while distorted, were recognizable, and general relationships were intact. Good preservation was judged by the presence of homogeneous nucleoplasm, and intact nuclear, cytoplasmic, and mitochondrial membranes. Conversely, the poor preparations possessed coarsely clumped cytoplasm and nucleoplasm, disintegrated mitochondria, and ruptured cellular membranes.

(CR refers to crown–rump length.)

Fig. 1.—Portion of an immature Malpighian corpuscle, illustrating loose arrangement of cells, lack of definite differentiation of parietal and visceral layers, and absence of a complete definite limiting basement membrane about the cell mass. Specimen fixed approximately 2½ hours post-mortem. *BM*, Basement membrane. CR 180 mm; weight 500 g.

Fig. 2.—Higher magnification of cells in another portion of corpuscle seen in Fig. 1. Note the relatively good preservation of cell structures, considering the intervention of 2½ hours between death and fixation. CR 180 mm; weight 500 g.



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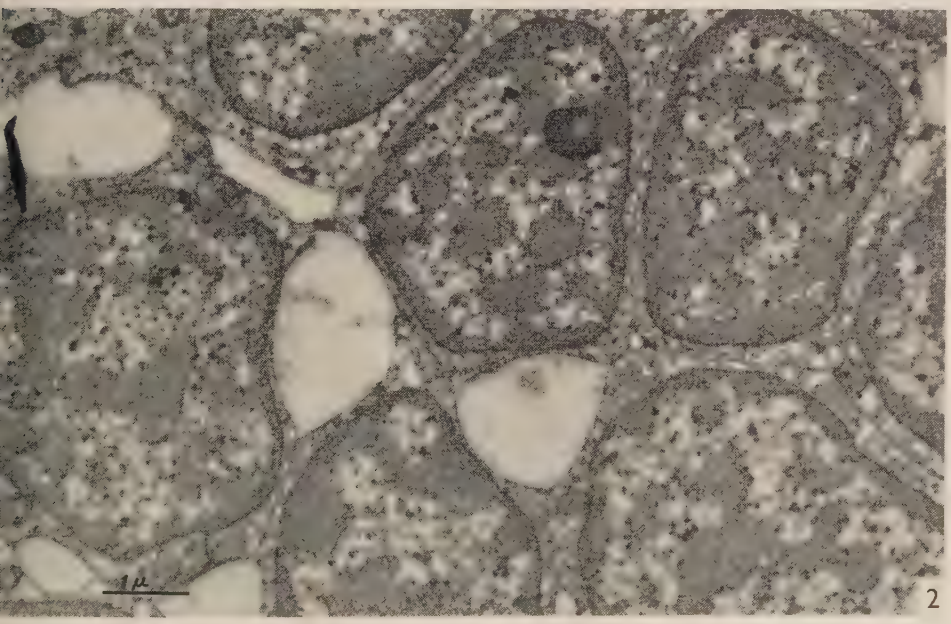

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Malpighian Corpuscle

The earliest recognizable Malpighian corpuscle consists of a loosely arranged mass of undifferentiated cells—each cell connected to several of its neighbors (Figs. 1 and 2). A small amount of amorphous material, seemingly primitive basement membrane, spreads about the edge of the cell mass, but there are many areas where this substance cannot be seen interposed between the cell mass and its neighboring developing renal tissue. As the developing glomerular tuft becomes evident adjacent to the cell mass, a cleft develops in the mass, resulting in the major share of cells becoming intimately inter-related with the developing glomerular tuft, while a single layer of cells remains on the opposite side of the cleft (Fig. 3).

This latter group can now be considered the parietal portion of Bowman's capsule. For a period of time its cells assume a cuboidal shape with no striking distinguishing characteristics (Fig. 3). Later, as the developing glomerulus enlarges, these cells appear to cease multiplying, and become stretched and thinned in order to cover the entire parietal aspect of the growing corpuscle.

The migrating cell mass destined to become the visceral layer of Bowman's capsule quickly differentiates in appearance from that of the parietal layer. Its cells are characterized by an increased density of nuclear and cytoplasmic proteins, numerous vacuoles throughout the cytoplasm, abundant poorly defined mitochondria, and occasional traces of Golgi substance (Figs. 4, 6, 7). The cell borders are sharply defined from one another. The most striking feature of these cells, however, is the presence of a great number of finger- or foot-like extensions of the cell surfaces at all points where they abut the glomerular tuft. The over-all appearance is one of crowding and pushing, each cell attempting to establish a "foot-hold" on the capillary anlage. Sections deep in this capillary anlage reveal that in each space and on every surface that the anlage presents, these primitive visceral epithelial cells

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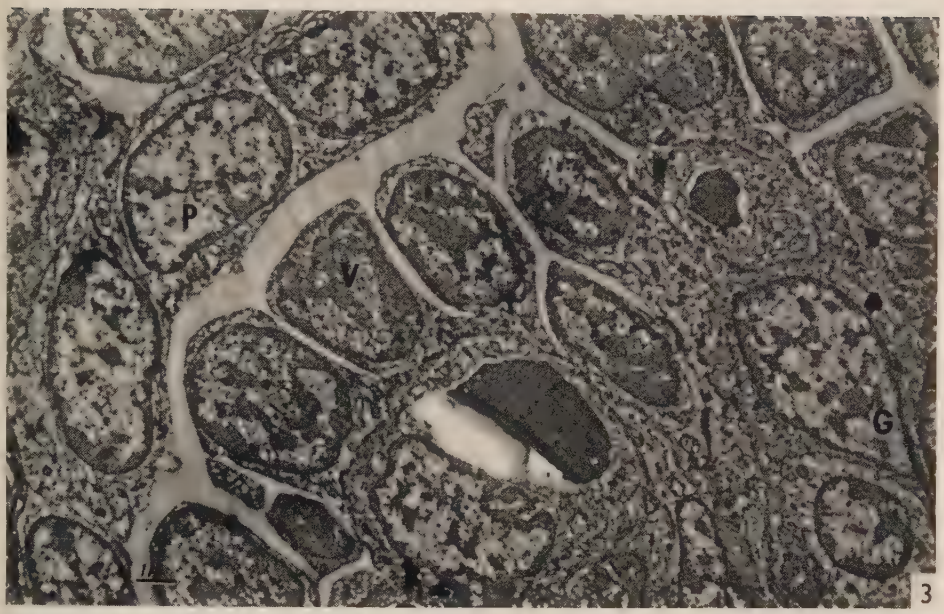
Fig. 3.—Separation of visceral cells from parietal cells. Note that the parietal cells are still cuboidal. The expanding glomerular tuft is seen at the lower right. CR 270 mm; weight 1500 g. *P*, Parietal cells; *V*, visceral cells; *G*, glomerular tuft. Specimen fixed 4 hours after death.

Fig. 4.—Section includes parietal cells, visceral cells and glomerular anlage. Note the difference in appearance between parietal and visceral cells, aside from the foot processes of the visceral cells. A delicate basement membrane is seen between the visceral cell foot processes and the sprouts of the primitive glomerular tuft. The capsular basement membrane is poorly defined. Within the developing glomerular tuft there are two red blood cells, with the beginning of a lumen present to one side of them. A granular cell of unknown nature is present in another projection of the tuft. Specimen fixed within one hour after death. CR 200 mm; weight 620 g. *P*, Parietal cell; *V*, visceral cell; RBC, red blood cell; *BM*, basement membrane; *G.C.*, granular cell.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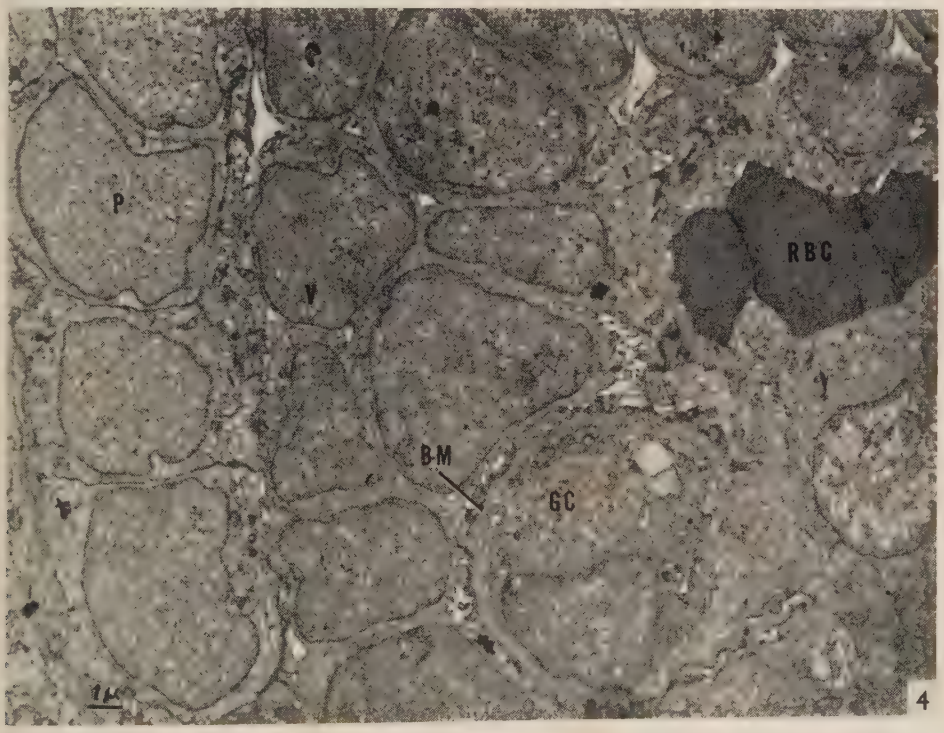
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migrate and apply their foot-like extensions (Fig. 5). The foot processes contain a dense, homogeneous substance which under high magnification appears to be derived from mitochondrial disintegration (Fig. 6). The cleft, or capsular space is almost obliterated by the crowding of elements (Fig. 4). At no time is there seen a large hollow cavity which subsequently is invaginated by developing capillaries.

Glomerular Capillaries

The primordial cell group which is destined to become the capillary tuft is seen simultaneously with differentiation of the visceral from the parietal portions of the Bowman's capsule. These cells possess ill-defined boundaries which appear to blend indistinctly with a somewhat dense homogeneous ground substance. The nuclei are often the only clearly identified parts of the cells. There is no differentiation between cells, and the appearance is that of a mosaic of nuclei, embedded in an amorphous substance which is interrupted in many places by the intrusion of foot processes of the primitive epithelial cells (Fig. 5). The highest order of differentiation of this mass, regardless of the stage of the development of the total glomerulus occurs in those cells forming the "froni" with the actively infiltrating epithelial cells. The arrangement, analyzed by studying many sections, is one of growth of cells in solid sheets and cords into the epithelial cell mass. A thin basement membrane is evident at all points where the capillary anlage apposes the epithelial cell foot processes.

Differentiation of the cells composing the growing primitive capillary tuft occurs in two directions. One is to produce endothelial cells, which vary only slightly in appearance from the anlage cell type. The other cell type that develops *in situ* in the cords are blood cells, and in particular, red blood cells (Figs. 4 and 6). The stages in the development of this latter cell

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Fig. 5.—Section deep in the cell mass of the early glomerular tuft. Note the almost syncytial appearance of the primitive endothelial and blood cells. All defects in the cell mass are filled with foot processes of visceral epithelial cells. The basement membrane is poorly defined throughout the mass of tissue. Specimen fixed within one hour after death. CR 200 mm, weight 620 g. *FP*, Foot processes; *V*, visceral cell; *RBC*, red blood cell; *BM*, basement membrane.

Fig. 6.—Section through the hilum of a glomerulus, showing transition between parietal and visceral epithelium, and blending of delicate glomerular tuft basement membrane with that of capsule basement membrane (arrow). The dense substance in the epithelial cell is seen with no surrounding lumen. The peculiar bleb-like structure to the left of the blood cell is observed regularly in relation to blood cell formation. It may represent a stage in development of the lumen. Note the two sets of double membrane structures within the basement membrane at the right. Specimen fixed within one hour after death. CR 200 mm, weight 620 g. *P*, Parietal cell; *V*, visceral cell; *RBC*, red blood cell; *FP*, foot processes; *BM*, basement membrane.

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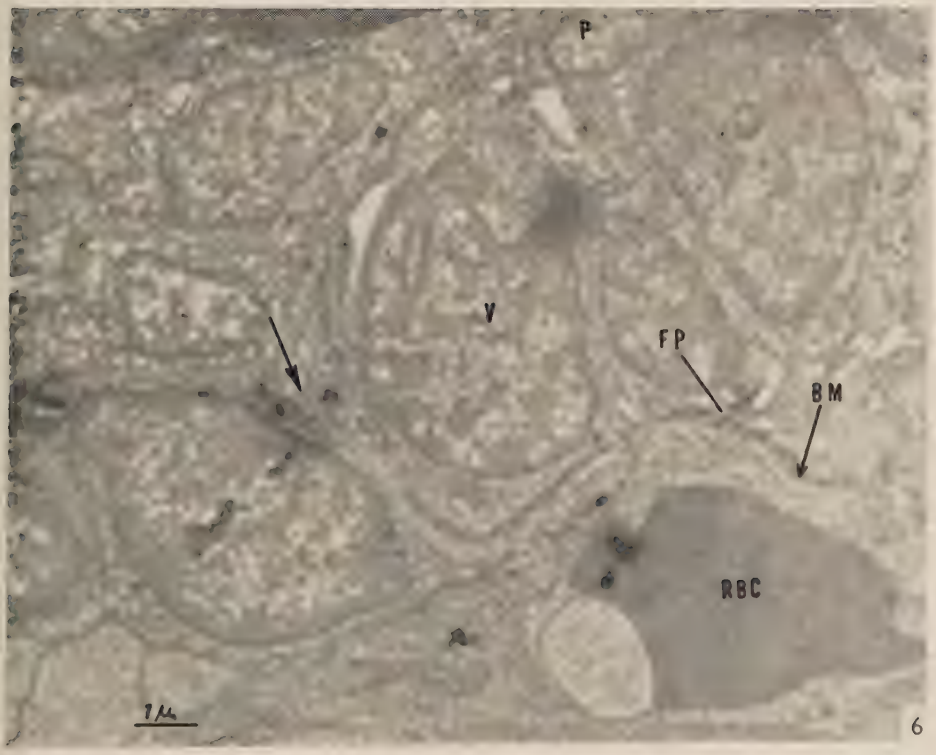
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type cannot be distinguished. For example, in none of the preparations could a normoblast be identified. Throughout all of the youngest glomeruli, this phenomenon is present. What appears to be completely mature red blood cells are embedded in the mass of primordial endothelial and hemopoietic cells, their limiting membranes distinctly separate, but closely applied to the cell membranes of the nucleated cells immediately adjacent (Fig. 6). As growth progresses, space gradually develops about these red cells, apparently by retraction and thinning of the endothelial lining, and soon, the isolated areas of lumen evidently link up to form a continuous vascular channel. Differentiation of these young vessels to the general appearance of adult glomerular capillaries goes rapidly to completion, long before extensive arborization of vessels takes place (Fig. 7).

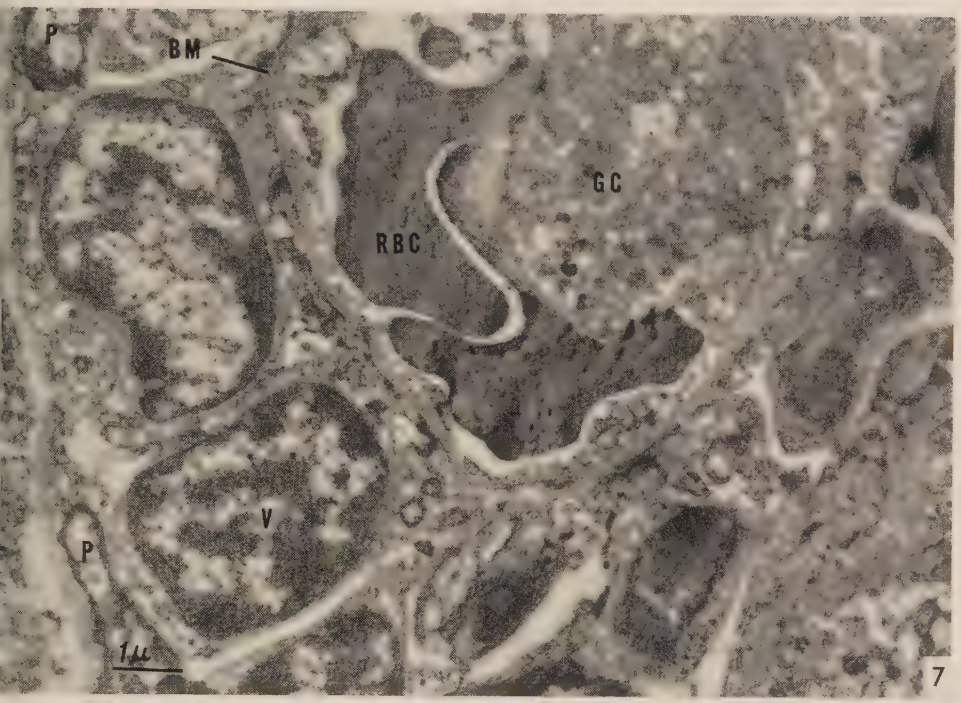
Glomerular Basement Membrane

In this description, the term "glomerular basement membrane" or "basement membrane" will refer to the electron dense layer between endothelial lining and epithelial cell processes, the "lamina densa" of the mature glomerulus [6].

As was noted in the previous sections, the early Malpighian corpuscle was seen to be at least partly surrounded by a homogeneous appearing ground substance, thicker in some places, but extremely sparse in most places, while the primitive hemopoietic and endothelial cells are seen to be embedded in a substance of similar appearance. As was noted also, at the earliest sign of apposition of primordial glomerular tuft with visceral epithelial cells, a well-defined but thin layer of electron-dense material is seen to be interposed. This represents the early basement membrane of the mature glomerulus. At low magnification, the earliest basement membrane is hardly visible, while that occurring in regions of more advanced development is sharp, clear, and dense. High magnification reveals that the early basement membrane contains structures which cannot be regularly perceived in the more advanced stage of development. The area between the cell surfaces is completely filled with an amorphous, moderately electron dense material, and embedded in this material are two sets of double membrane structures,

Fig. 7.—Section showing development of lumen about a group of blood cells in the early glomerular tuft. CR 200 mm; weight 620 g. *P*, Parietal cell; *V*, visceral cell; *GC*, granular cell; *RBC*, red blood cell; *BM*, basement membrane. Specimen fixed within one hour after death.

Fig. 8.—Section illustrating the presence of two sets of double membranes within the glomerular basement membrane (arrows). A lumen is present about the single red blood cell. CR 240 mm; weight 1000 g. RBC, red blood cell; *E*, endothelial cell; *V*, visceral cell.


P
BM
GC
RBC
V
P
1μ
7

E
RBC
V
1μ
8

one set close to the epithelial cell surface, the other close to the endothelial cell surface, with a wide space between the sets (Figs. 6 and 8). It would appear that these double membranes rapidly lose most of their electron density, and blend into the amorphous material, only occasionally leaving a suggestion of these membranes in the older basement membranes.

In fortuitous sections that go through the region where the parietal wall of Bowman's capsule becomes continuous with the visceral layer, the aforementioned parietal basement membrane is seen to blend smoothly with that of the glomerular membrane (Fig. 6). At times a double membrane can be seen in the parietal basement membrane, but never is there more than one set of membranes visible.

Recognizable connective tissue fibers cannot be seen in any phase of development in the glomerulus.

DISCUSSION

The present observations confirm the concept, first clearly stated and adequately supported by Herring [7], that the glomerular capillaries develop as solid cords in the mass of cells on the concave side of the developing Malpighian corpuscle. He quotes Bornhaupt [3], Thayssen [17] and Riedel [16] as reporting similar observations. These same investigators noted that the cavity of the Malpighian corpuscle developed from a split occurring in the cell mass, in contradistinction to the view held by Remak [15] and Toldt [18]. These latter investigators reported that the glomerulus developed by invagination of a hollow vesicular Malpighian corpuscle, created by the upgrowth of capillaries which then become surrounded by the capsular basement membrane. Huber [8] reported observations almost identical to those of Herring, and stated, as well, that the lobulation of the capillary tuft was produced by penetration of epithelial cells between the loops. Noel and Pigeaud [12], Okkels [13], and Gruenwald and Popper [5] published observations supporting the concept of invagination of a hollow vesical in glomerular development, while Vimtrup [20], and Loeffler [9] reported finding the situation as described by Herring. Apparently, the invagination theory proved more attractive to investigators of the adult glomerulus, for upon this they were able to describe the capillaries as supported by the invaginated capsular basement membrane in a sort of "sling" [10, 11] or "mesangium" [22].

The observations presented in this paper lend no support to the theory that the glomerular basement membrane is derived from the invaginated

capsular basement membrane. On the contrary, all observations point to the likelihood that capsular basement membrane is not involved in the process. It was noted that even after the appearance of the capillary tuft, the capsular basement membrane was poorly developed, and certainly was not present as a complete structure about the early corpuscle. It was noted, too, that the visceral cells of Bowman's capsule rapidly altered in appearance once the intervening cleft of capsule space had separated them from those cells destined to form the parietal layer.

The basement membrane which is interposed between these newly differentiated visceral epithelial cells and the primitive endothelial cells of the growing capillary loops is seen to contain two longitudinally oriented electron dense lines, each suggestive of double membranes, or remnants of double membranes. Since each set appears to be closely related to the epithelial and endothelial cells respectively, it is suggested that this mechanism is somehow involved in the production of basement membrane, and that the basement membrane represents a contribution from epithelial and endothelial cells. If these observations and assumptions are correct, then the invagination concept becomes untenable.

The curious concept of Trabucco and Marquez [19] that the glomerulus develops as multiple evaginations of a capillary-like sinus into blind pouches rather than into loops or networks also receives no support from the observations presented here.

The observations regarding the phenomenon of blood cell formation *in situ* still leave much to be desired. At no time can a recognizable stem cell of the red blood cell series be identified, while apparently mature red cells are seen regularly, embedded in the cellular material of the developing capillary tuft long before a lumen is evident. Occasionally, cells with well-defined granules in their cytoplasm are seen in this developing cell mass, but their identity cannot be established. Okkels [13] reported the phenomenon of *in situ* red cell formation in the developing glomerulus, and claimed to have observed normoblasts and other cells of the series. Noel and Pigeaud [12] reported no evidence of this type of blood formation, and stated that the capillaries of the glomerulus were empty before they were united with the arciform vessel outside the glomerulus. On the basis of identifying only one morphologically distinct parent cell in the developing glomerular tuft, this work supports Okkel's concept that the mesenchymal cell mass involved in tuft formation has a toti-potential quality as regards endothelial cells and blood cells. Aschoff [1] reported similar findings to those of Okkel's in the adult kidney as well as in the fetal glomerulus.

The electron microscopic appearance of the differentiated visceral epithelial cells is in accord with that seen in the adult glomerulus as reported by numerous investigators. In the case of the fetal kidney, however, the demands of growth have not yet reached the point where the cytoplasm and foot processes of these cells have become attenuated, and these cells therefore fill almost all of the potential capsular space.

The perforations of the endothelial lining, again visualized in the electron micrographs of mature glomeruli by numerous investigators, are seen only rarely in the fetal glomerulus. This is related to the fact that in the preparations observed in this work, the endothelial lining rarely becomes attenuated to the degree necessary for the fenestrations to occur.

Aside from blood cells, mesenchymal cells, and endothelial cells, no other cell type can be recognized in the developing tuft. No fibrillar structures resembling connective tissue are present in any of the glomeruli studied. This fact again causes us to scrutinize carefully the concepts of connective tissue networks supporting the glomerulus [21, 2, 10, 11, 22].

SUMMARY

1. Electron microscopic observations of developing human renal glomeruli are presented.

2. The Malpighian corpuscle develops as a mass of epithelial cells in continuity with the convoluted tubule. It is surrounded by a sparse, incomplete layer of basement membrane material.

3. Capsular space develops by cleft formation when the primitive glomerular tuft appears, leaving a single layer of Bowman's capsule on one side.

4. The cells that develop into visceral epithelial cells rapidly differentiate in appearance from the parietal cells by developing a more dense protoplasm and cytoplasmic foot processes. Foot processes are present only where the cells are apposed to the developing glomerular tuft.

5. The developing capillary tuft originates as broad sheets and cords of endothelial-like cells, and appears to be shaped by penetration of the visceral epithelial cells. There is no sign of invagination of a hollow vesicle in any preparation.

6. The glomerular basement membrane is a product of endothelial and visceral epithelial cells, and appears to receive a contribution in the form of double membranes from each.

7. Fully matured red blood cells are observed frequently, before capillary lumens are present. This is taken to indicate *in situ* development of red

blood cells in the glomerulus. It is suggested that the capillary lumen develops about these red cells by retraction and shrinking back of endothelial cytoplasm.

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GLASS SUBSTRATE CULTURES OF HUMAN BLOOD AND BONE MARROW CELLS

H. J. WOODLIFF

Department of Medicine, University of Cambridge, England

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The culture of cells on inert substrates such as glass and in direct contact with nutrient fluids is now a well established technique in studies on cellular morphology and physiology and on the response of cells to infectious agents [2].

Established cell strains and cells derived from embryonic tissue are commonly cultured in this manner. Blood and bone marrow cells are more often studied by nutrient substrate and fluid deposit methods but some studies on glass substrates have also been made. Weiss and Fawcett [11] found the method suitable for their study of the cytochemical changes associated with the transformation of chicken monocytes into macrophages, epithelioid cells and multinucleated giant cells and Berman [1] employed it for long term tissue culture of human bone marrow.

This paper describes my evaluation of the method as a result of a larger study of blood and bone marrow cell cultures.

MATERIALS AND METHODS

240 cultures and 54 subcultures of cells derived from 43 specimens of bone marrow fluid and 7 specimens of blood taken from patients with various haematological and other disorders were studied.

These included cells from nine patients with leukemia, two with leukemoid reactions, five with carcinomas, two with myelomatosis and two with sarcomas as well as several with different types of anaemia.

Specimen collection and preparation of the inoculum. A few drops to two millilitres of bone marrow fluid were aspirated from the sternum, vertebral spine or ilium and collected into one 1 ml of human serum containing one 1 mg of heparin. In most cases marrow flecks were removed from the marrow aspirate for culture by other methods. The remaining fluid was then diluted further with human serum and medium 199 [4] before inoculation.

Cells from the peripheral blood were collected by venipuncture and partial separation of the leucocytes from the erythrocytes was made by a variety of methods such as natural sedimentation, the use of phytohaemagglutinin [6] and cold centrifugation.

Fig. 1.—Saunders tube cultures on rack.

None of the methods tried was completely satisfactory as a large red cell contamination was always found. The cells were usually diluted with serum and medium 199 before being set up in culture.

In some experiments a nucleated cell count was made and the inoculum adjusted to between 1 and 4 $\times 10^6$ cells in each millilitre.

Culture medium and gas phase.—A mixture of normal serum (20 per cent) and medium 199 (80 per cent) was used routinely. Culture fluids were replenished as required; usually half the old medium was discarded and a similar volume of fresh added.

In a few experiments medium 199 alone was used and in others 6-mercaptopurine dissolved in sodium hydroxide and then buffered with medium 199, was added.

In most of the cultures the gas phase was air. Some cultures were made in gas phases of oxygen with 5 per cent carbon dioxide and of nitrogen.

Culture vessels.—Most of the cultures were made in Saunders [9] tubes (supplied by S. H. Travis & Co., 296/300 St. John Street, London, E.C.1). The tubes were 25 mm in diameter, 15 ml in capacity and were closed by screw caps lined with silicone rubber washers. They were chosen for their ease of handling and relative cheapness. The thin neutral glass has reasonable optical properties allowing low, but not high, power microscopical examinations of the living cells to be made. Each tube was inoculated with 1 ml of cell suspension and incubated at 37°C lying horizontally on a special rack (Fig. 1). A few cultures were also prepared in culture chambers (Fig. 2). In the Harris [3] chambers the inoculum was allowed to settle in the smaller bottom chamber and then the middle coverslip was removed allowing the fluid to come into contact with the gas phase in the upper chamber. The dimensions of the chambers were chosen so that the proportion of fluid nutrient to gas phase would approximate that found in the Saunders tubes. In the Rose [8] chambers there was no gas phase apart from a few small bubbles of air remaining after inoculation.

Methods of observation. Cells in the Saunders tubes were observed by inspection

and microscopically by means of the × 10 objective and × 8 eyepieces of a C.T.S. microscope fitted with a long range condenser. It was not possible to get phase contrast illumination because of the relatively large diameter of the culture tube. During observation the tube had to be turned through 180° for the cells to be brought near the objective; as this also removed them from the nutrient fluid the observation time was limited. Photomicrographs were taken with a quarter plate camera. A few of the cultures were fixed and stained; photomicrographs of these were taken through × 12 objective and × 8 eyepiece of a Leitz ortholux microscope fitted with a 35 mm camera.

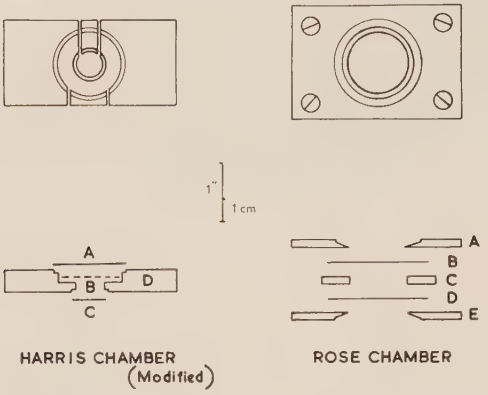


Fig. 2.—Diagram of culture chambers. *Harris chamber: A*, top coverslip; *B*, middle coverslip; *C*, bottom coverslip; *D*, perspex body. The coverslips are sealed onto the body with paraffin wax and the chamber inoculated through the drill holes which are then closed by polythene plugs. *Rose chamber: A*, top stainless steel plate; *B*, top coverslips; *C*, white rubber gasket; *D*, bottom coverslip; *E*, bottom stainless steel plate. The plates are joined by screws and the chamber inoculated through hypodermic needles inserted through the rubber.

Several methods of marking the tubes for cell counting were tried. The most suitable method was found to be the scratching of a circle with a diamond (Watson object marker) on the outside of the tube. The size was adjusted so that the circle just filled the field of a special eyepiece fitted with a graticule. The number of cells either in the whole field or in a portion measured by the graticule were then counted. Cells in the Harris chambers were examined microscopically with both × 10 and × 75 objectives of a C.T.S. microscope. By filling the larger chamber with fluid medium the cells could be observed with phase contrast illumination but the definition was rather poor. In some experiments the coverslips were removed and the cells fixed and stained for study by ordinary microscopy. Cells in the Rose chambers were observed both by low (× 10) and high (× 70 and × 75) power objectives with phase contrast illumination on both C.T.S. and Leitz microscopes.

Methods of subculture. -Subcultures were only attempted in the Saunders tube cultures. Removal of the supernatent fluid into another tube either with or without scraping the growth with a polythene rod was used in some experiments. In other experiments the cultures were washed in serum-free medium 199 and then incubated with Bacto-Trypsin solution. (Bacto-Trypsin solution final dilution equivalent to 0.25 per cent of Trypsin Difco 1:250) for periods of 5 minutes or more. At the end of the incubation period the trypsin was neutralised with fresh serum and the cells washed with fresh medium before inoculation.

Subcultures were also made with the aid of ethylenediamine tetracetic acid (EDTA, versene or sequestrene). The cultures were first bathed and incubated in a magnesium

nd calcium free salt solution. A solution of disodium EDTA to give a final dilution f 0.05 to 0.1 per cent was then added and allowed to act for 5 minutes or longer. After centrifugation at 4°C the cells were resuspended in their original medium.

RESULTS

An overall pattern of culture divisible into three successive but overlapping ytological phases was observed in the Saunders tubes. Cells of the first hase were those recognisable as types normally present in blood or bone narrow. The only cells which could be definitely identified were polymorphonuclear leucocytes which were present for up to 5 days and megakaryocytes (Fig. 5) a few of which survived for up to 30 days. Monocytes nd myelocytes could not be distinguished at the low power of magnification. Lymphocytes and erythroblasts do not adhere to glass surfaces and were ot therefore seen.

Cells of the second phase will be called large granular cells (Fig. 4). They were usually recognised at 2 or 3 days and persisted in decreasing numbers for or 3 weeks. For example, in one culture in which serial counts were made f these cells in six circles the average numbers were 100 at 3 days, 75 at 5 days, 33 at 6 days, 30 at 7 days, 26 at 12 days and 15 at 14 days. In some ultures occasional cells survived for up to 40 days.

The cells were mostly rounded and fairly large (20–40 μ in diameter); ome were polygonal and a few spindly. The cytoplasm was generally granular and obscured the nucleus. In some cells a spreading hyaloplasm ormed a halo around the granular plasm. Hypertrophy of the cells to 100 μ iameters was seen in some cultures. No evidence of the multiplication of these ells was found.

Cells of the third phase resembled fibroblasts (Figs. 3, 4, 6, 7 and 8). hey were found in the majority of the bone marrow cell cultures but not n the cultures of peripheral blood cells. A few cells of this type were usually ound between 6 and 20 days. After being present for several days there ppeared to be a rapid multiplication into sheets and colonies. The cells ersisted for between 60 and 120 days before dying out. Morphologically hey resembled the fibroblasts of classical histology.

No difference in the pattern of culture attributable to the clinical condition f the patient from whom the cells were derived was detected.

Medium 199 without serum would not maintain the cells. They died ithin 1 to 3 days when the routine medium was replaced by medium 199 lone.

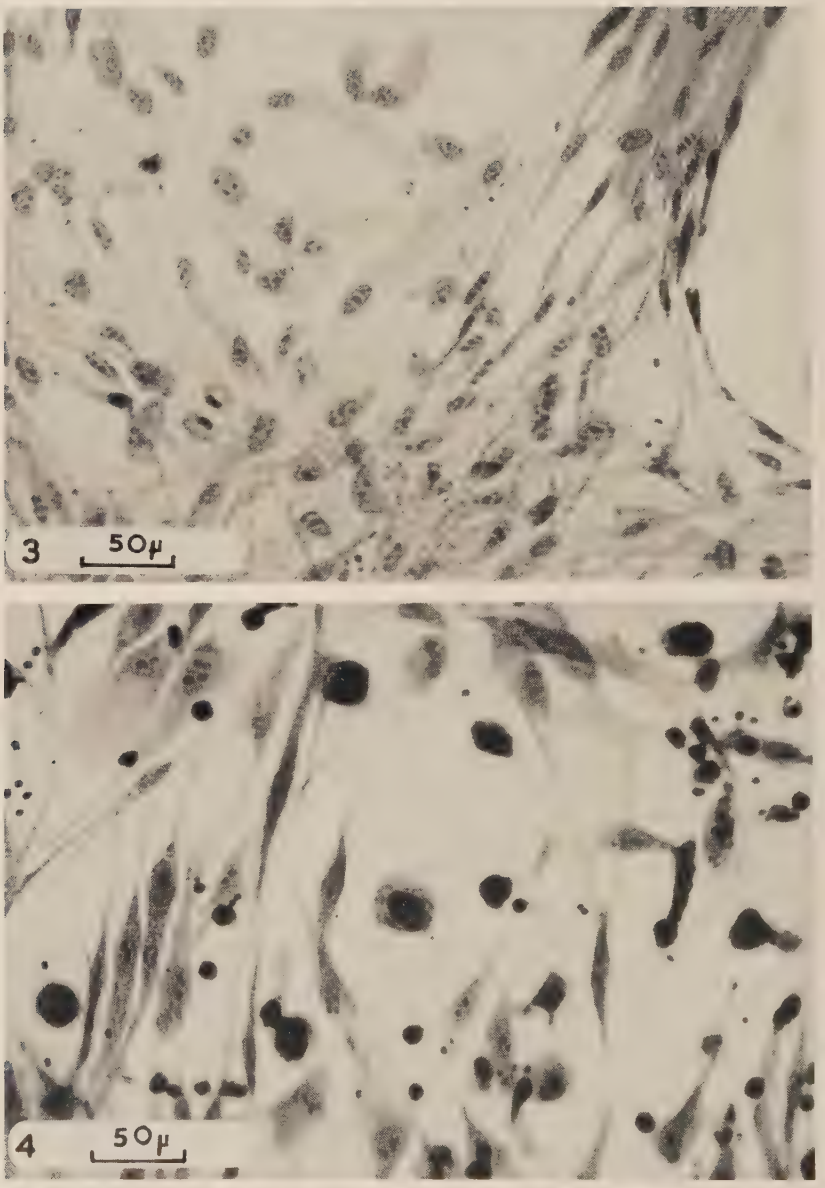
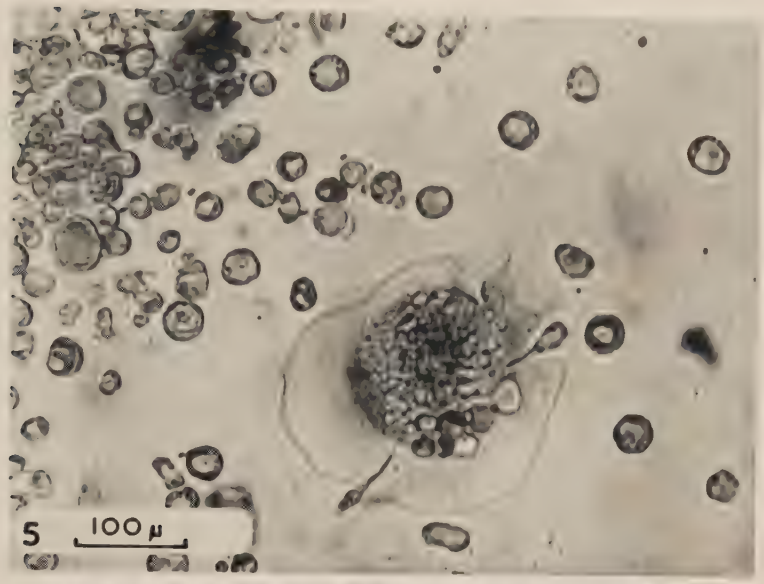


Fig. 3. Sheet of fibroblast-like cells in a 15-day Saunders tube culture of bone marrow cells. An anaphase is present towards the bottom left. Fixed and stained by Leishman.

Fig. 4.—Large granular and fibroblast-like cell in a nine-day Saunders tube culture of bone marrow cells. Fixed and stained by Leishman.



g. 5.—Megakaryocyte and large granular cells in a 7-day Saunders tube culture of bone marrow ls. Living cells photographed with ordinary illumination.

g. 6.—Fibroblast-like cells growing out of a small fleck of bone marrow in a 12-day Saunders be culture. Living cells photographed with ordinary illumination.

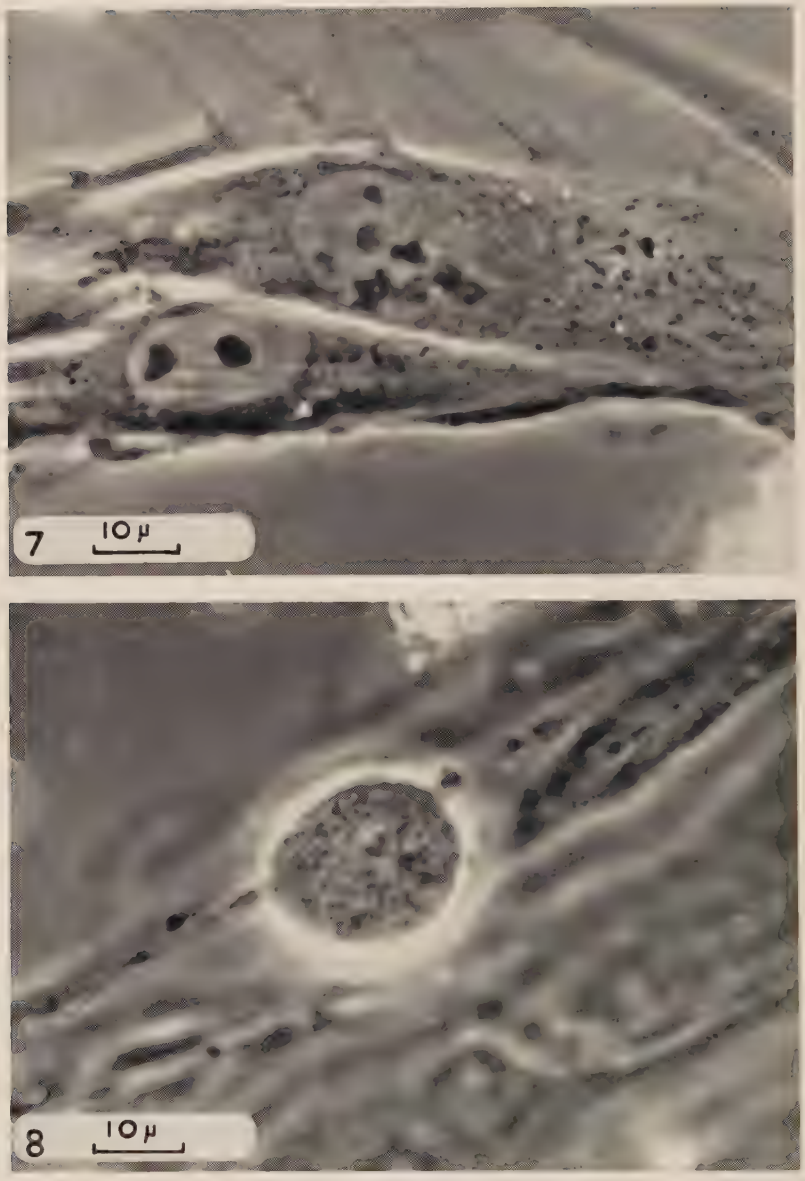


Fig. 7.—Fibroblast-like cells in a 5-day Rose chamber culture. Living cells photographed with phase contrast illumination.

Fig. 8.—Metaphase of a fibroblast-like cell in a 5-day Rose chamber culture. The other cells in the field are attached to the glass surface above the focal plane of the chromosomes in the rounded mitotic cell. Living cells photographed with phase contrast illumination.

In one experiment gas phases of air, oxygen with 5 per cent carbon dioxide nd nitrogen were compared. The normal pattern was seen in the cultures ith an air gas phase. In cultures with a gas phase of oxygen with 5 per cent arbon dioxide there was an accelerated decrease in the number of large anular cells and fibroblast-like cells did not develop. In the cultures with itrogen as the gas phase the round granular cells showed the usual pattern ut relatively few fibroblast-like cells were seen and they survived for only a w days.

The effect of 6-mercaptopurine upon the cultures was studied in several xperiments. No effect was observed in a concentration of 1 millimole per ml ut concentrations over 6 millimoles per ml prevented the normal pattern f culture. Concentrations between 1 and 6 millimoles per ml gave variable ffects.

Subcultures were made by removing the supernatant fluid, by scraping ie growth off the glass with a polythene rod, by trypsinisation and by the se of EDTA. Although viable cells were obtained by each of these methods one were suitable for quantitative work with replica cultures, since clumps s well as single cells resulted. The yield of separated cells after removal f the clumps was too low to be of value. The use of trypsin or EDTA for eriods long enough to complete disaggregation caused cell death.

Subcultures of the supernatant fluid during the first five days gave rise large granular cells and to fibroblast-like cells in some experiments.

Cultures in the Harris and Rose Chambers were poor as compared with ose in Saunders tubes. The cells did not survive in many of the cultures nd no consistant pattern emerged. The chambers did, however, allow high ower phase contrast microscopy of the individual cell types and were herefore better for morphological studies. The Rose chamber was found be superior to the Harris chamber both for general handiness and for icroscopy.

DISCUSSION

The three cytological phases found in these cultures confirm the findings f Berman [1] and correspond to those found in other types of blood and one marrow cultures. Cells of the first phase are more conveniently studied y solid nutrient substrate and fluid methods. Cells of the second phase, ie large granular cells, probably correspond to the histioid and macrophage ells seen in other types of culture [1]. As the adjective histioid might be taken mean that the cell is derived from connective, as opposed to haemopoetic, ssue, it has been avoided. The origin of the cells is not clear and it is quite

possible that they are a transformation of more than one pre-existing cell type. The pre-existing cells must be present in fair number in the marrow and in peripheral blood, and for this reason fixed connective tissue cells cannot be a major source. The monocyte appears to be an obvious choice, but since large numbers of the cells were present in some cultures in which pre-existing monocytes were not recognised, other possibilities must be considered. Cells of the myeloid series for example might be so transformed; a possibility which could only be confirmed by continuous long term observations on a single cell. The term macrophage has not been used because evidence of phagocytosis was not seen in the cultures. The presence of large quantities of granular material in the cells might be taken as evidence of such activity and might also result from the metabolic activities of cells imbibing soluble nutrients.

Cells of the third phase, the fibroblast-like cells resemble the fibroblasts seen in histological sections of connective tissue. There was no evidence of fibre formation however and it is probable that cells of this kind as seen in culture represent several different types. The fact that they appeared in marrow cultures but not in those of peripheral blood is in favour of a connective tissue origin. As they multiply rapidly they might originate from similar cells present but unrecognised in the original material. Their formation by transformation of haemopoetic cells cannot however be excluded and continuous observations will be necessary to clear up this point. Similar cells have been observed in other types of culture and in flecks of marrow cultured on serum agar they appear to migrate from the centre [7, 12]. This also suggests a connective tissue origin.

No diagnostic value could be found in this series of cultures. Berman [1] who isolated a polygonal strain of cells (Detroit-6) from the bone marrow of a patient with a carcinoma of the lung suggested that the cultures might provide opportunities for detecting metastasis in patients with occult tumours. In one of the present series of experiments numerous carcinoma cells were seen in a smear of the inoculum but could not be recognised in the cultures. Also the possibility of polygonal cell strains arising by transformation of pre-existing non-malignant cell types makes the cultures valueless in this respect. Berman [1] also suggested that the cultures might be useful in the study of myeloproliferative disorders involving the connective tissue elements. Since it is extremely difficult to obtain satisfactory bone marrow aspirates from patients with these disorders this possibility is remote.

Large numbers of human fibroblast-like cells are regularly obtained in the glass substrate cultures of bone marrow aspirates and they therefore

orm a convenient source of such cells for morphological, physiological and pharmocological studies.

Preliminary experiments with nutrient fluid without serum, different gas phases and 6-mercaptopurine show that the cultures are suitable for semi-quantitative studies similar to those of Neuman and McCoy [5] who used chick embryo cells.

More elaborate techniques based on replica subcultures of cells must wait further refinements in techniques of disaggregation.

SUMMARY

Methods and results of culturing human blood and bone marrow cells on glass substrates are reported.

A pattern of culture divisible into three successive but overlapping phases was observed. Cells of the first phase, recognisable as normal blood cells, are more suitably studied by other techniques.

The large granular cells of the second phase and the fibroblast-like cells of the third phase are described and their origin discussed. Glass substrate cultures of bone marrow cells are a convenient and reliable source of human fibroblast-like cells for morphological, physiological and pharmocological experiments. They have no value as an aid to diagnosis.

This work was carried out during the tenure of an Elmore Studentship. I am grateful to Dr. F. G. J. Hayhoe for advice and help with the manuscript, to Mr. R. J. Clemans and Miss T. Minett for photographic and technical assistance and to the Melville Trust for the loan of a microscope.

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SOME ASPECTS OF THE STRUCTURE OF THE INNER ROOT SHEATH OF HAIR FOLLICLES REVEALED BY LIGHT AND ELECTRON MICROSCOPY

G. E. ROGERS

Biochemistry Unit, Wool Textile Research Laboratories, C.S.I.R.O., Parkville, Victoria, Australia

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The inner root sheath of hair follicles is composed of concentric cylinders of cells, namely the Henle and Huxley layers, and the cuticle. In wool follicles for example, each of these layers is one cell thick. In larger follicles however, they may be two cells in thickness. These cells are originally cuboidal in shape in the bulb of the follicle, but they become elongated and thin as they move up with the growing hair.

Histological descriptions of the inner root sheath as it appears in the light microscope after sectioning and staining have recently been given by Auber [1] and Montagna [8]. In the present paper some further features of its structure are presented. Information on the shape and arrangement of the constituent cells and on their ultrastructure has been obtained by two techniques, namely shadow-casting for revealing surface texture under the light microscope, and ultra thin sectioning for examination by electron microscopy.

METHODS

The inner root sheath layers in the follicles of sheep, guinea pigs and young mice were studied.

Shadow-casting for light microscopy.—Wool roots with their inner root sheaths intact were removed from sheepskin by the method of Ellis [4], suspended in 0.25 *M* sucrose and then agitated in a Waring blendor for 2 min. This procedure removed much of the unhardened tissue from the wool roots whereas in many cases the hardened layers of the inner root sheaths were still attached and could be distinguished under the light microscope.

These preparations were washed by centrifugation and portions dried on microscope slides for shadow-casting with gold-palladium by a method described earlier [5]. Photomicrographs were taken on Ilford plate emulsion R.40. Prints were obtained after reversals had been prepared.

Electron microscopy. Pieces of skin from adult guinea pigs and 5-day-old mice were fixed for 4 hr. in OsO_4 buffered to pH 7.4 [9]. After methacrylate embedding

butyl methacrylate containing 5 per cent methyl methacrylate), ultrathin sections of contained hair follicles in appropriate orientation, were cut on a microtome [7]. They were examined at 80 kv. in a Siemen's Elmiskop I fitted with a 25 μ objective aperture.

RESULTS

Shadowed specimens.—At follicle levels corresponding to the zone of prekeratinization in the cortex, shadowed areas of Henle's layer from wool roots have the appearance shown in Fig. 1. The Henle layer has opened out into a sheet of interconnected and flat cells with its innermost aspect exposed (in an intact follicle this surface adjoins the Huxley layer). The cells have nearly reached their final hardened state.

Fig. 2 is from a portion of the field shown in Fig. 1, at higher magnification. Several details of structure can now be seen. The plaque-like cells are 30–40 μ long and 20–30 μ wide and are very thin; the lengths of their shadows show that they are about 1 μ in depth.

At their lateral junctions (*r*, Fig. 2) adjacent cells do not overlap greatly although distinct ridges are present, indicating sites of close contact between the cells.

The cell boundaries are indented at both ends of each cell. At these edges, which are distal and proximal with respect to the direction of growth, overlap is more extensive. For example in Fig. 2, the distal edge of cell *a* lies astride the proximal edges of two preceding cells. However the overlap is not complete and a small space about 4 μ wide remains. The intercellular gaps shown in Fig. 2 are indicated by solid arrows. This pattern of cell arrangement is a regular feature of the Henle layer, and is not an artefact induced by factors such as drying. Thus intercellular gaps could be recognised in undried specimens examined in aqueous suspension.

Several flattened nuclei with oval outlines can be seen in the cells shown in Fig. 2. Although some appear centrally placed in the cells, others are in more proximal position. The fibrous texture of the cells suggests the presence of fibrous components in their cytoplasm. The striations which can be observed, are oriented parallel to the direction of growth.

Fig. 3 shows several cells of Huxley's layer at a level in the wool follicle similar to that of Henle's layer shown in Fig. 2. At this stage they appear to be much flatter than the corresponding Henle cells. Striations within their cytoplasm again suggest the presence of oriented fibrous components. Nuclei can also be identified. Ridges exist at the lateral junctions between cells, but areas of overlap as in the Henle layer are not present.

Fig. 4 is a photomicrograph of part of the cuticle of the inner root sheath of a wool follicle. It is readily seen that the surface of this sheath of extensively overlapping and flattened cells is a replica of the cuticle of the wool fibre with which it is in contact in the follicle. Again, outlines of ovoid nuclei can be distinguished. Their major axes are at right angles to the direction of movement of the cells in the follicle.

Electron microscopy.—As they move up from the bulb, the cells of the inner root sheath undergo transformations which can clearly be seen in electron micrographs.

At early stages of differentiation the cytoplasm of these cells is similar to those of presumptive hair cells. Few or no membranes of endoplasmic reticulum were observed but some small mitochondria were present in a granular ground-cytoplasm. The granular inclusions of trichohyalin develop in the cells of the inner root sheath. They are electron-dense and vary greatly in size (Figs. 5, 6 and 7). At the same time however, fine filaments about 50 Å in diameter also appear within the cytoplasm (Figs. 5, 6 and 7). They are oriented parallel to the long axes of the cells and thus also to the direction of movement of the inner root sheath. Tufts of these filaments together with granules of trichohyalin were observed in cross-sections of the cytoplasm of Henle cells of young mouse follicles as shown in Fig. 5. On the other hand longitudinal sections as in Fig. 6 revealed the tufts of filaments extending along the lengths of the cells.

The filaments increase in quantity until they fill the cytoplasm of the cells, whereas other components such as the trichohyalin granules, mitochondria and the granular ground-cytoplasm disappear. Of particular interest is the suddenness with which the final appearance of filaments takes place, and this is well shown in Fig. 7. In Henle cell *a* which is from the follicle of a guinea pig, the filaments are loosely arranged in the cytoplasm, and enmesh several small trichohyalin granules. In cell *b* the cyto-

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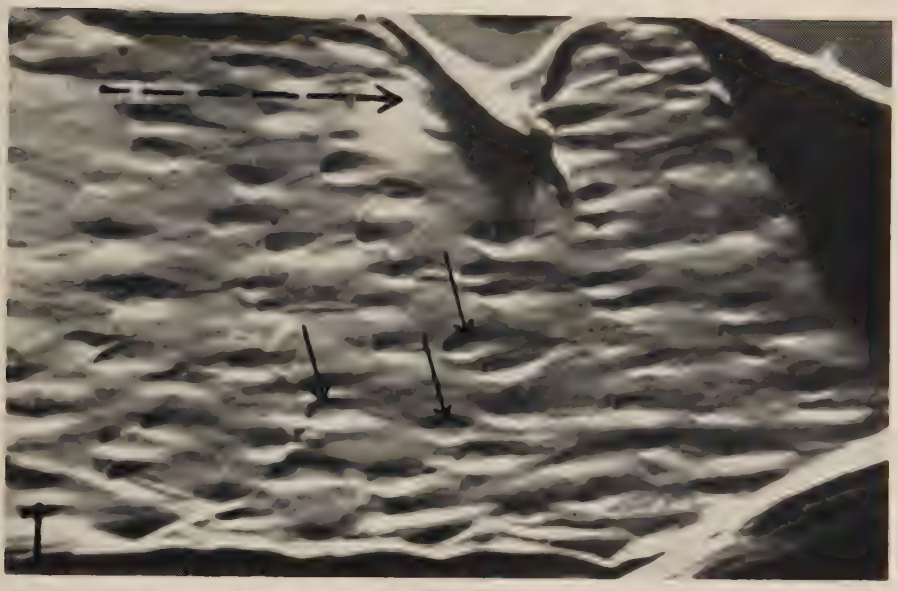
Abbreviations.—*fil*, filaments; *He*, Henle layer; *Hu*, Huxley layer; *ic*, inner root sheath cuticle; *N*, nucleus; *ORS*, outer root sheath; *r*, ridge; *tri*, trichohyalin. Dotted arrows indicate the direction of upward movement of the cells in the follicle.

Fig. 1.—The inner surface of Henle's layer in a wool follicle showing the overlapping plaque-like cells. Small gaps are present between some of the cells, three of these are indicated by arrows. Shadowed with Au Pd, achromatic objective × 10. Magnification × 450.

Fig. 2.—Part of Fig. 1 at higher magnification showing four cells *a*, *b*, *c* and *d*. Areas of contact between cells *a*, *b* and *c*, *d* can be seen. The intercellular gaps are indicated by arrows. Note the ridges at the lateral junctions between the cells. Shadowed with Au–Pd. Fluorite objective × 54. Magnification × 1500.



a
c
N
N
r
r
b
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N
2

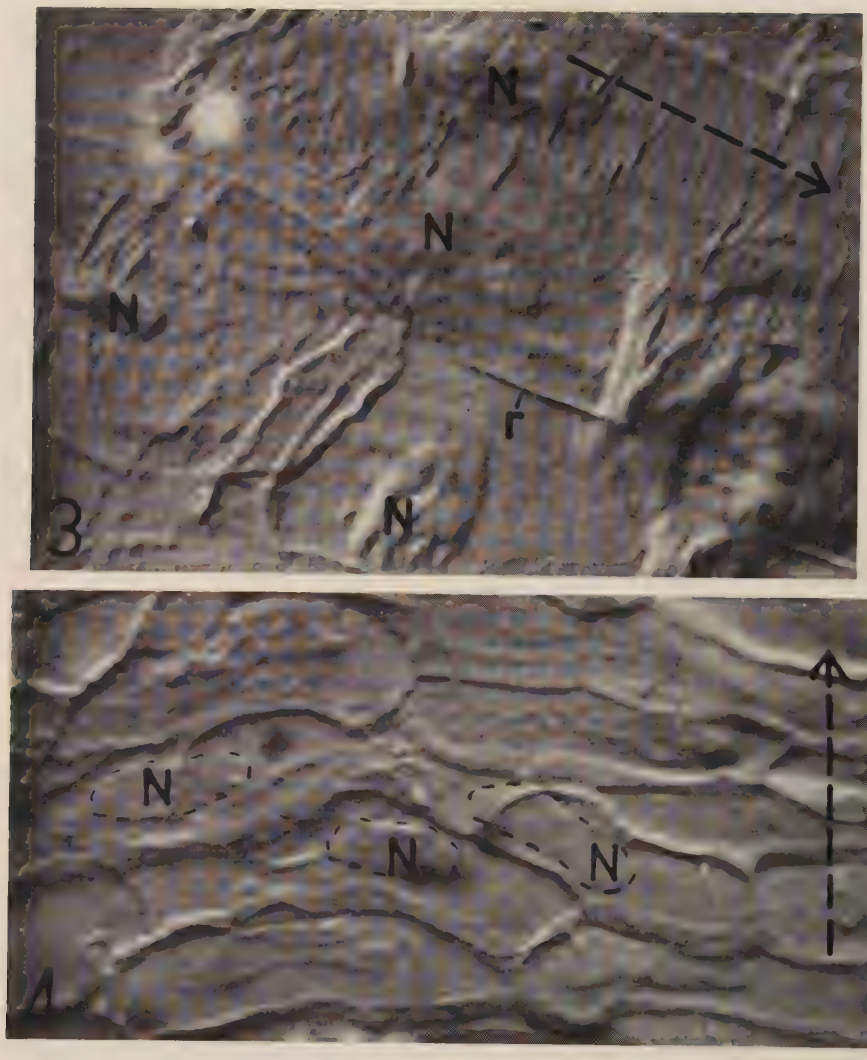


Fig. 3.—The flattened cells of Huxley's layer in a wool follicle. Note the ridges at the lateral junctions between cells, and the nuclei. Shadowed with Au Pd. Fluorite objective 54. Magnification × 1680.

Fig. 4. The flattened cells of the inner root sheath cuticle. They take the form of a replica of the cuticle of the wool fibre with which they were in contact. Note the elongated nuclei. Shadowed with Au–Pd. Fluorite objective × 42. Magnification × 1300.

Fig. 5.—Cross-section of a hair follicle of a 5-day-old mouse. In the Henle layer, dense trichohyalin granules and tufts of filaments are present in the cytoplasm of the cells. In the Huxley layer the trichohyalin granules are smaller and filaments have not developed. Note the cell membranes (cm). Electron micrograph, × 14,000.

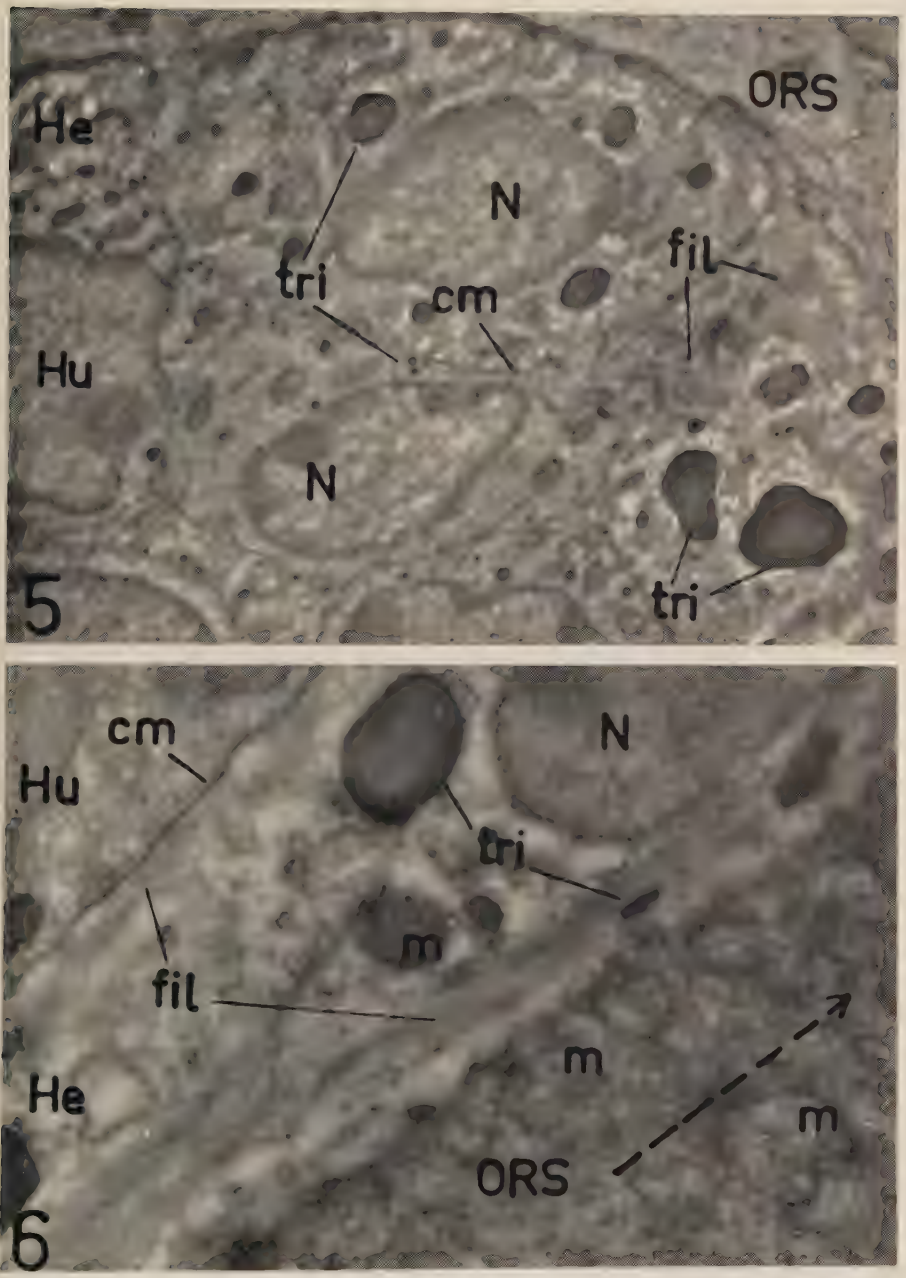
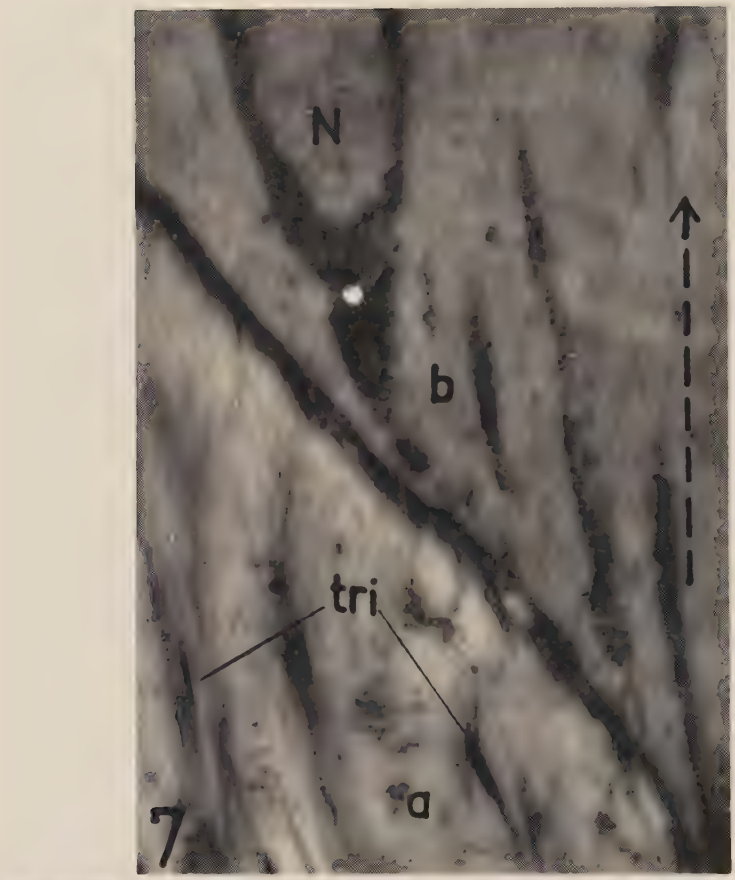


Fig. 6.—Longitudinal section through a hair follicle of a 5-day-old mouse. Note the dense boundary (*cm*) between the Huxley and Henle cells. Trichohyalin granules are enmeshed by filaments in the Henle layer, and a mitochondrion (*m*) is present. Several mitochondria are to be seen in the outer root sheath cell. Electron micrograph, × 24,000.


N
b
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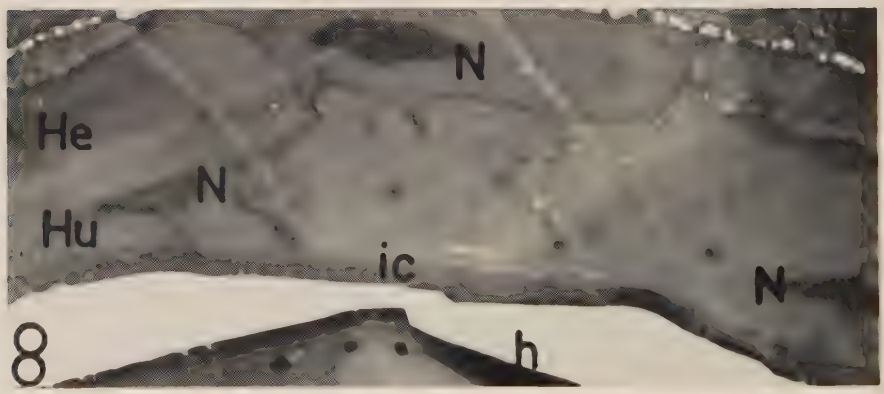
a
7


N
He
N
Hu
ic
N
8
h

plasm is packed with filaments but trichohyalin granules are no longer to be found.

It appears that the filaments coalesce and harden still further, since at higher levels of a follicle the cells of the inner root sheath present a homogeneous appearance (Fig. 8). In addition, within this hardened material, electron-dense, usually flattened inclusions are often seen (Fig. 8). These are remnants of nuclei.

Although the changes here described were illustrated by reference to Henle's layer, they also occur in a similar way in the cells of Huxley's layer and of the cuticle of the inner rooth sheath.

DISCUSSION

The microscopic examination of metal-shadowed specimens of inner root sheath reveals particularly well the shape and arrangement of the constituent cells. Of interest are the intercellular gaps which occur between adjacent cells of the Henle layer. From Fig. 2 it is seen how the cellular arrangement can give rise to them. It should be noted that Auber [1] quotes Brunn [3] as being the first to demonstrate that these gaps are not artefacts. Indeed in the present work they were observed by phase-contrast microscopy of preparations before drying down. However it is only after shadow-casting that the cellular pattern and intercellular gaps can be so clearly revealed. Nutrients and metabolic products could pass through these gaps between the follicle and the growing hair. The cell processes from the Huxley layer called Flügelzellen [8] must therefore protrude through these gaps in active follicles.

The fact that the layers of the inner root sheath which have been examined were not readily disrupted into their constituent cells suggests that the union between cells was fairly strong. Apart from the observation of the points of contact between Henle cells in shadowed specimens, and the fact that the intercellular membranes were relatively dense in electron micrographs (Figs. 5, 6 and 8), no other features of intercellular contact were seen. However, other workers [2] have demonstrated by high resolution electron micro-

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Fig. 7.—Longitudinal section through two cells (*a*, *b*) of the Henle layer of a hair follicle from a guinea pig. This micrograph illustrates the suddenness with which the filaments (approx. 50 Å wide) finally appear. In cell *a* these filaments are loosely arranged around trichohyalin granules, whereas in cell *b* the cytoplasm is packed with filaments. Electron micrograph, × 19,000.

Fig. 8.—Cross-section through the hardened inner root sheath layers and part of a hair (*h*) of a follicle from a guinea pig. Individual hardened cells which are outlined by the denser cell boundaries, contain nuclear remnants. Electron micrograph, × 4500.

scopy, that the cell membranes of inner root sheath cells are separated by a distance of 250–300 Å and that within this space is contained a complex-layered structure of dense material. This material presumably is the agent responsible for binding the cells together. Similar modifications of membranes in other epithelial cells have been observed [11, 12].

From the examination of shadowed specimens it was noted that cell nuclei are usually but not always in the proximal regions of the cells of Henle's layer. The identification of dense areas as nuclei in electron micrographs of the hardened cells of the inner root sheath (Figs. 7 and 8) is supported by the fact that the nuclear remnants never lose their ability to give a positive Feulgen reaction [6].

The fibrous nature of the intracellular content of the hardened cells of the inner root sheath was revealed by the electron microscope (Figs. 5, 6 and 7). The filaments which finally fill the cells are about 50 Å in diameter, and morphologically resemble the microfibrils of the hair cortex, although they do not undergo the intermediate organisational stage to macrofibrils. They are the submicroscopic elements which are responsible for the increase of birefringence which takes place [6] during the growth of the inner root sheath.

Histochemical studies of the inner root sheath [6] indicate that the filaments consist of protein. Their rapid formation occurs whilst trichohyalin, mitochondria and the granular ground-cytoplasm disappear. These changes progress until enzymes of the Krebs cycle are no longer detectable [10] and the cells die. Trichohyalin may be reabsorbed [1] or it might be converted into filaments. If the latter, then the rapid formation of filaments could be explained despite the lack of components such as mitochondria which are usually considered obligatory for protein synthesis.

It is not known, however, how the isotropic trichohyalin granules are chemically related to the filaments. Thus for the moment it is not reasonable on morphological evidence alone to suggest the direct transformation of one into the other.

Chemical and histochemical studies of the proteins of the inner root sheath would be of interest in relation to this problem, and are being pursued in this Laboratory.

SUMMARY

The cells of the inner root sheaths of hair follicles have been studied by the techniques of metal shadowing for light microscopy, and ultrathin sectioning for electron microscopy.

The shapes and interrelationships of the cells of the different layers are described. The presence of well-defined gaps between adjoining cells of Henle's layer is discussed.

Electron microscopic examination revealed the disappearance of mitochondria and trichohyalin granules from these cells, whilst intracellular filaments about 50 Å in diameter are formed.

It is not known whether trichohyalin granules are intermediates in the formation of these filaments. Chemical evidence is required in addition to the morphological features described.

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ELECTRON MICROSCOPIC INVESTIGATIONS ON THE LEPTOMONAD FORM OF *LEISHMANIA DONOVANI*

C. K. PYNE

Biophysics Division, Institute of Nuclear Physics, Calcutta, India

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Since the pioneer investigations of Emmel, Jacob and Geolz [9] on *Leishmania donovani*, this protozoon has been the subject of a number of electron microscopic studies, including those of Das Gupta, Bhattacharya and Sen Gupta [6] and Das Gupta, Guha and De [7]. More recently Chang [5], using the technique of ultrathin sectioning, has studied the intracellular form besides the flagellate form of this species. Löfgren [16] has studied the allied species, *Leishmania tropica*. The present paper reports some further investigations carried out on the flagellate form of *L. donovani*, with the help of the electron microscope.

MATERIAL AND METHODS

The leptomonad form of *Leishmania donovani* was cultured in N.N.N. (Novy, McNeal and Nicolle) medium in this laboratory. Cultures 8 to 12 days old were used for the present studies.

The flagellates were washed out of the culture tubes by normal saline, and washed further with normal saline to remove the agar particles and other impurities. They were fixed for one hour in a 1 per cent solution of osmium tetroxide, buffered with acetate veronal to pH 7.4 [18] and then dehydrated through the alcoholic series. All these changes were affected through centrifugation at 3000 rpm for 3 min. Embedding was done in a 1:4 mixture of methyl methacrylate and butyl methacrylate, which was polymerised at 48°C for 24 hrs. For sectioning, a modified Spencer Rotary Microtome, Model 821, was used along with glass knives. The sections were collected on the surface of acetone–water mixture (1:3) and stretched on dioxane–water mixture (1:1). They were then mounted on grids previously coated with collodion membrane and examined under the electron microscope without removal of the embedding medium.

A few specimens were treated after fixation with distilled water for 24 hrs and mounted directly on collodion membranes. This technique, though not in much use now, was found to be of some use in the present studies.

A Siemens Elmiskop I was used at 60 kv for observation. Electron micrographs were taken at a magnification of about 10,000, except in a few cases, and then enlarged photographically as desired.

OBSERVATIONS

The pellicle or periplast.—The body of the protozoon is covered by a well defined pellicle or periplast which is seen in sections to consist of two distinct layers (Fig. 4). The total thickness of the pellicle is about 400 Å. The outer pellicular layer is smooth, continuous and thin, being less than 100 Å. The inner layer is made up of a series of fibrils, each of which appears to be 150 Å in diameter in sections. These pellicular fibrils are visible clearly in sections passing somewhat tangentially through the pellicle (Fig. 3). They are separated from each other by a mean distance of about 200 Å. The pellicular fibrils are also clearly seen in specimen treated with distilled water after fixation (Fig. 1). Here they measure about 250 Å in diameter, with a mean separation distance of 200 Å. This discrepancy is obviously due to the swelling and/or flattening inherent in this procedure. In distilled water-treated material, the pellicular fibrils further show indications of a regular periodicity along their length, which however is not apparent in sections. The mosaic pattern of pellicular fibrils observed in Fig. 1 is due to the superimposition of the two sides of the flattened body.

At the point of origin of the flagellum, the outer pellicular layer continues over the flagellum as the flagellar sheath, while the inner pellicular layer folds inside to form a tube-like sheath surrounding the intracellular parts of the flagellar fibrils (Figs. 5–7).

The nucleus.—The nucleus is visible in most of the sections as a well defined body, circular, oval or elliptical in shape, depending on the plane of the section (Figs. 3 and 4). The nuclear membrane is formed of two distinct layers. The outer nuclear membrane is thin, being only 100–125 Å thick, while the inner nuclear membrane is much thicker, being 400–500 Å thick, and more osmiophilic. Inside the nucleus one or more, densely osmiophilic, variously shaped bodies are visible. A maximum of five such bodies have been counted. When single, it may represent the karyosome or endosome; but when more than one body is visible, it is more likely that these represent sections through the chromosomes. Sen Gupta and Ray [26] described six chromosomes in this species. As to the ultrastructure of these bodies, no regular details can be discerned in osmium-fixed material.

Mitochondria.—The mitochondria appear in sections as variously shaped bodies dispersed in the cytoplasm (Figs. 4 and 7). They are not visible in all the sections, obviously due to the differences in the plane of the sections. They are limited by a well defined mitochondrial membrane, about 100 Å thick. Whether the mitochondrial membrane is double or not, cannot be

discerned at the levels of resolution obtained here. Inside the mitochondria, structures of various sizes and shapes appear, which presumably represent sections through the tubular projections of the mitochondrial membrane, termed "microvilli" by Sedar and Porter [25].

The flagella.—The flagellum is found to consist of a flagellar sheath and a number of fibrils running longitudinally along its length. The flagellar sheath is very thin, being about 100 Å only, and, as already stated, is a continuation of the outer pellicular layer. In transverse section of the flagella (Fig. 8) one central fibril is seen surrounded by nine peripheral fibrils. The central fibril is slightly thicker than the rest, having a dimension of 450 Å × 250 Å in this section, where some amount of compression has obviously occurred. The nine peripheral fibrils average about 350 Å × 250 Å. In one of the sections (Fig. 6), where the flagellum has been cut longitudinally in its proximal region, a maximum of six fibrils can be counted, with a mean diameter of about 200 Å. In *Leishmania* treated with distilled water after fixation (Fig. 2) the flagellar fibrils are clearly seen running parallel to each other and in a bundle. Here, also, the diameter of each of these fibrils appears to be about 200 Å. These fibrils seen in Figs. 2 and 6 are therefore much thinner than the fibrils seen in Fig. 8, and may well represent the subfibrils constituting the former: as a matter of fact, the protozoan cilia has been reported to be formed of ten pairs of fibrils, one central and nine peripheral, the members of each pair being in close apposition ([20, 21, 25]; see Discussion).

Basal apparatus of the flagella.—The flagellar fibrils extend into the body of the organism for a distance of about 1–1.25 μ (Fig. 5). As mentioned above, they are surrounded here by a sheath formed by the invagination of the inner pellicular layer. The fibrils terminate in swollen granular structures, which lie near the parabasal body; no connexion has yet been found, however, between these fibrils or granules and the parabasal body. The latter is a transversely elongated, densely osmiophilic body, measuring 0.4 0.7 μ in length and about 0.1 μ in breadth. It is surrounded at some distance by a membrane, although the space in between is not quite clear, as is to be

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Abbreviations.—*FF*, flagellar fibrils; *FS*, flagellar sheath; *INM*, inner nuclear membrane; *IP*, inner pellicular layer; *K*, karyosome; *M*, mitochondria; *N*, nucleus; *NM*, nuclear membrane; *ONM*, outer nuclear membrane; *OP*, outer pellicular layer; *P*, pellicle; *PB*, parabasal body; *PF*, pellicular fibrils.

Fig. 1.—Electron micrograph of a specimen treated with distilled water after fixation, showing the pellicular fibrils. × 25,000.

Fig. 2.—As in Fig. 1, showing the flagellar fibrils in two flagella. × 40,000.

PF
1μ
1
FF
1μ
2

expected if this area is the eosinophil vacuole visible optically. The membrane enclosing the parabasal body is about 200 Å thick.

Besides the above mentioned cellular organelles, various other smaller particulate or otherwise well defined structures of various shapes can be detected in the various electron micrographs, scattered in the cytoplasm. They range in size from 2000 Å downwards. Their exact nature is still obscure, though they have been variously described by other authors as lipoid granules, volutin granules etc. Further work is needed to elucidate the nature of these bodies.

DISCUSSION

The submicroscopic structure of various cell organelles observed here resembles more or less those observed by other authors in the same or allied species of Protozoa.

The pellicle or periplast shows the fibrils observed in this species by Das Gupta *et al.* [7], by Löfgren [16] in *L. tropica*, by Saxe [24] in *Euglena gracealis* and by others in various species of *Trypanosoma* [14, 15, 17]. Chang [5] observed them in ultrathin section of the Leishman bodies but not in the leptomonad forms, which is explained by the fact that these fibrils are visible clearly only in sections passing somewhat tangentially through the body surface. The present studies, however, reveal that these fibrils form an essential component of the pellicle and as such should be termed "pellicular fibrils" and not myoneme fibrils [7]. Indeed, these fibrils show no structural resemblance to the myoneme fibers of ciliates studied by Randall [20]. If any comparison is to be made at all, these pellicular fibrils may be compared to the fibrils constituting the myoneme fibers of ciliates. The contention of Meyer and Porter [17] that these fibrils are contractile and are responsible for body movements may still be true, in view of the periodicity observed by us as well as by the above authors in *T. equiperdum*.

The nuclear membrane of *L. donovani* has been found to differ from that of most other material studied so far. Although it has been found to be made up of two distinct layers, in the present studies as well as in those of Chang [5], there is no evidence of its having the porous or annular structure commonly found in other animal and plant material [4, 8] and even in other protozoa [3, 11, 13, 23]. Though it is not unlikely that a porous or annular

Figs. 3–8.—Electron micrographs of sections in various planes through the body and the flagella.

Fig. 3.—The pellicle at the top has been cut tangentially and shows the pellicular fibrils. The double nature of the nuclear membrane is also apparent. × 60,000.

PF
ONM
N
INM
P
1 μ
3

structure may be found on further investigations, the nuclear membrane of a flagellate has never yet been reported as having such a structure.

The size and distribution of mitochondria found here agree with that found by Guha, Pyne and Sen [12] through cytochemical studies in this material. Chang [5], however, failed to observe any definite mitochondria in sections of *L. donovani* observed by her. The ultrastructure of mitochondria found here is similar to that found by Porter and his collaborators in various protozoa [22, 25], having a mitochondrial membrane and a number of microvilli embedded in the mitochondrial matrix. Obviously this is one of the common types of mitochondrial structure occurring in Protozoa, although other types of structures have also been observed in some Protozoa. Thus Randall [20] found two types of mitochondria in ciliates: one type consisting of parallel sheets, 150 Å thick, separated by less dense regions, the other consisting "partly of sheets as above and partly of small tubular vesicles". These vesicles apparently correspond to the microvilli. Anderson [1] found the mitochondria of *Trichomonas muris* to "consist of a series of compartments". Powers, Ehret and Roth [19] described the mitochondria of *Paramecium* as consisting of "an osmiophilic continuum interrupted by spaces continuous with the cytoplasm".

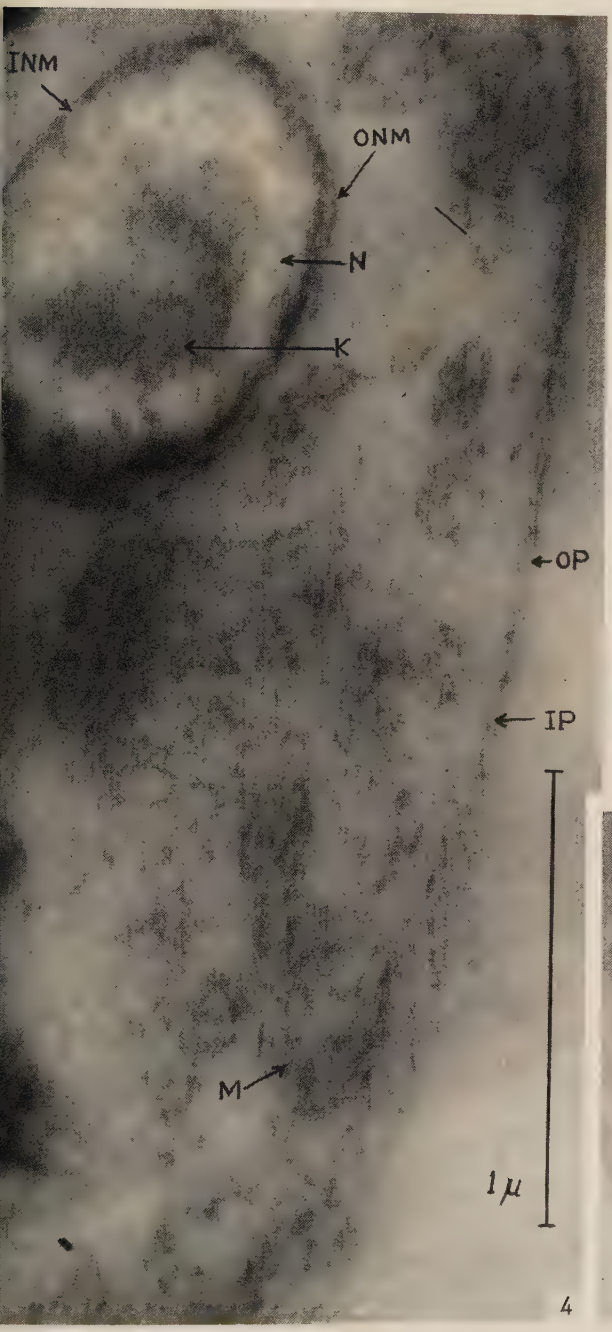
The flagellar structure shows the same pattern as found in other flagellates and ciliates, metazoan cilia, animal sperm tail and plant flagella. It can easily be assumed now that this pattern of one central and nine peripheral fibrils is universal to all such locomotory structures. The central fibrils have often been found to be double, and recently Fawcett and Porter [10], Sedar and Porter [25], Roth [21] and Randall [20] found that the nine peripheral fibrils are similarly double. The present studies also give some evidence in favour of the double nature of these flagellar fibrils. No evidence has been found for the presence of "a loosely coiled sheath" surrounding the flagellar fibrils reported by Chang [5] in both the forms of this species. The sheath enclosing the fibrils of cilia in *Euplotes* has been found to be double by Roth [21].

The basal apparatus of the flagellates is usually described as consisting of a parabasal body, a basal granule or blepharoplast and an eosinophil vacuole.

Fig. 4.—The double nature of the pellicle and the nuclear membrane is evident. Mitochondria are also visible. × 60,000.

Fig. 5.—The flagellum emerging from the body. The flagellar fibrils end near the parabasal body. × 50,000.

Fig. 6.—The infolding of the inner pellicular layer at the point of origin of the flagellum is clearly seen in this section passing through the proximal region of the flagellum. × 45,000.


INM
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OP
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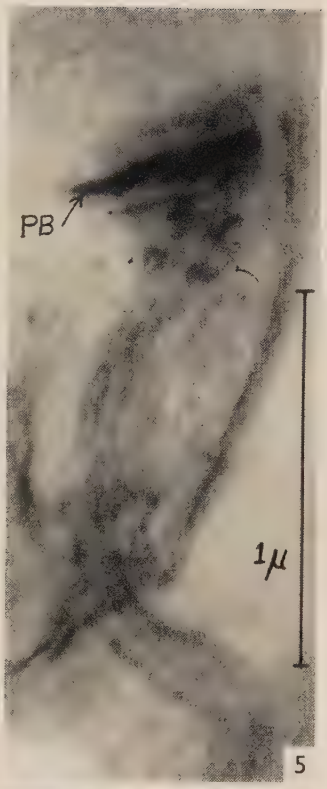
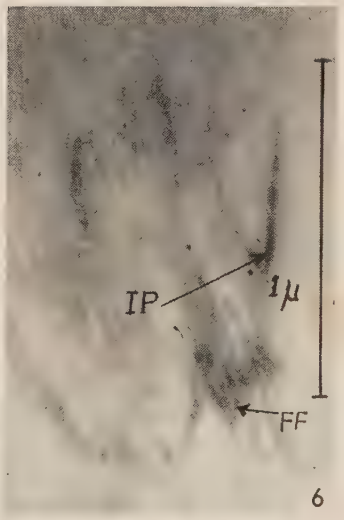
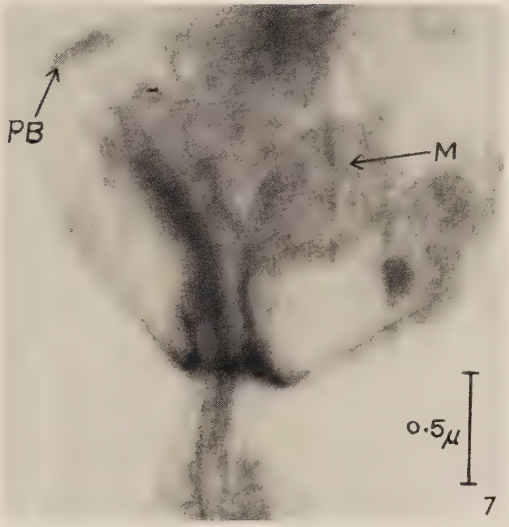
1μ
4


PB
1μ
5


IP
1μ
FF
6



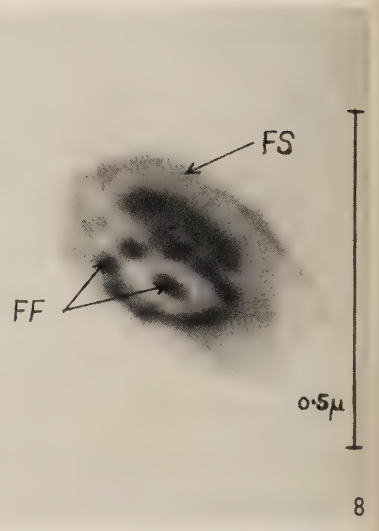


Fig. 7.—Section showing the parabasal body, the flagellum and a mitochondrion. × 30,000.

Fig. 8.—Slightly oblique section through the flagellum showing the arrangement of the flagellar fibrils. The nature of the body seen besides the fibrils is not clear. × 90,000.

The term Kinetoplast is used variously for the parabasal body or for the whole complex. In the present study, as well as in the previous studies on the same material and other allied flagellates, only one type of structure has been observed in electron micrographs of ultrathin sections, and that is the parabasal body. Chang [5], however, terms it "basal granule", though its elongated nature and transverse disposition leave no doubt that it is the same structure as that interpreted here as the parabasal body. Anderson *et al.* [2] call this body "kinetoplast". No separate basal granule has been found by us or by Chang [5] unless the granular swellings at the bases of the flagellar fibrils represent it. The structure termed "basal granule" by Anderson *et al.* [2] in *T. equiperdum* may well be a cross-section through the intracellular part of the flagellum. No distinct eosinophil vacuole has ever been observed, other than the space surrounding the parabasal body.

SUMMARY

1. The leptomonad form of the protozoon, *Leishmania donovani*, has been studied electron-microscopically after ultrathin sectioning, as well as after treatment with distilled water following fixation.

2. The pellicle has been found to be formed of two layers: an outer thin continuous layer and an inner thicker layer formed of parallel fibrils.

3. The nucleus is provided with a double nuclear membrane and inside ontains a number of distinct structures, which have been interpreted here s the chromosomes.

4. The mitochondria show the typical structure with a distinct membrane nclosing a system of microvilli.

5. The flagellum consists of ten fibrils running longitudinally along its ength. Each of these fibrils is probably formed of two subfibrils.

6. The flagellar fibrils terminate internally in granules which lie near the arabasal body. The latter is a transversally elongated body enclosed in a iembrane.

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DISTRIBUTION OF CYTOCHROME OXIDASE IN AN EARLY CLEAVAGE STAGE OF *DENDRASTER*

W. E. BERG

Department of Zoology, University of California, Berkeley, Calif., U.S.A.

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Localization of substances, particularly enzymes, has been found during early development of the ascidian, *Ciona* [1, 2]. A similar study was made of animal and vegetal cells of the sand dollar, *Dendraster excentricus*, to determine if localization of constituents might be correlated with metabolic or physiological gradients claimed to exist along the polar axis of echinoderm eggs and embryos.

Quantitative measurements of cytochrome oxidase, apyrase and ribonucleic acid were carried out; however, only results on cytochrome oxidase are reported here. Apyrase activity of animal and vegetal cells was too low to be measured accurately with the micromethod of Lowry *et al.* [8]. Ribonucleic aicd, measured by a micromethod based on the procedure of Ogur and Rosen [9], seemed to be more concentrated in mesomeres of the 16-cell stage; however, the results were too erratic to be conclusive.

Animal and vegetal cells of *Dendraster* cannot be distinguished until the 16-cell stage is reached; at which time the embryo, exhibiting typical echinoderm development, consists of mesomeres, macromeres and micromeres. Attempts to obtain isolated animal and vegetal cells at the 16-cell stage were carried out by progressive separation of blastomeres beginning at the two-cell stage. With this procedure, however, nearly all vegetal cells unexpectedly underwent equal cleavages and abnormally large micromeres were formed. Furthermore, isolated first cleavage blastomeres, even if left undisturbed, often produced vegetal cells that cleaved equally with atypical micromere formation. It appears that the unequal cleavage forming the micromeres is an extremely labile one, and it is particularly interesting that a disturbance of cytoplasm several cleavages prior to micromere formation often has a delayed effect on cleavage of the vegetal cells.

Because of the above difficulties it was necessary to separate animal and vegetal cells after completion of the fourth cleavage with all cleavages occurring under conditions as normal as possible. Eggs were inseminated and within three minutes transferred to 0.1 per cent protease in order to dissolve the fertilization membranes

TABLE I. *Cytochrome oxidase activity—(Δ log ferrocyt. c)/ΔT in homogenates of animal and vegetal cells from the 16-cell stage of* Dendraster.

Number of pairs	Cytochrome oxidase in mesomeres	Cytochrome oxidase in macromeres-micromeres	Ratio mac.-mic./mesomere
100	0.028	0.022	0.79
120	.041	.043	1.05
150	.127	.082	0.65
150	.064	.084	1.31
120	.042	.041	0.98
120	.046	.042	0.91
120	.045	.036	0.80
120	.037	.031	0.84
120	.062	.069	1.11
120	.076	.070	0.92
120	.053	.048	0.91
120	.086	.102	1.19
			Average 0.96 ± 0.05

nd jelly coats. After dissolution of the membranes the eggs were transferred to gar-coated dishes and left undisturbed until completion of the fourth cleavage. An investigation of various solutions and factors led to the use of 5 per cent protease n Ca-free sea water at pH 5 as being effective in causing disassociation of the blasto-neres. The solution was kept at a low temperature to prevent subsequent cleavages nd generally within one-half hour the cells became separated, a process which ould be accelerated by agitation.

Disassociation usually occurred with many blastomeres remaining together s pairs, i.e. two mesomeres together or macromeres with their companion micromeres. For convenience the cells were selected and counted as pairs, wo mesomeres being considered an equivalent amount of cytoplasm to ne macromere plus the adhering micromere. This is justified since the third leavage of the *Dendraster* egg is equal.

Cytochrome oxidase activity was determined by spectrophotometric meas-rement of the oxidation rates of reduced cytochrome *c* [5] with a Beckman spectrophotometer adapted for the use of microcuvettes [7]. The techniques or handling the cells and preparation of homogenates were similar to those described previously [1].

Values for cytochrome oxidase activity, expressed as velocity constants, f homogenates of animal and vegetal cells are listed in Table I. These are elative values of enzyme activity and due to different conditions for each

set of paired measurements, the data are expressed as ratios of enzyme activity in animal and vegetal cells. With such minute amounts of material the homogenization procedure is critical and undoubtedly accounts for the large error of measurement. Twelve paired measurements of cytochrome oxidase activity in homogenates of vegetal cells averaged 96 per cent that of animal cells, a difference which is not significant.

The data indicate no marked localization of cytochrome oxidase which might be responsible for the physiological gradients of Child [4]. There is, however, the possibility that a cortical gradient of the enzyme might be present which, involving perhaps a fraction of the total amount, would not be easily detected by quantitative measurements of total enzyme activity.

Although cytochrome oxidase is, in a variety of cells, intracellularly localized in mitochondria, the evidence is conflicting for localization of this enzyme in echinoderm eggs [3]. Assuming a mitochondrial localization of this enzyme the data are in agreement with Gustafson and Lenicque [6] who find, by direct mitochondrial counts, no mitochondrial gradient in earlier stages.

Child [4] states that the gradient of dye reduction becomes more pronounced in later stages. Furthermore, Gustafson and Lenicque [6], by direct mitochondrial counts, describe a mitochondrial gradient in blastulae, a result questioned, however, by Shaver [10]. Particularly in view of these claims an attempt was made to determine whether differences in cytochrome oxidase might occur in embryos developing from animal and vegetal regions.

Animal and vegetal cells of the 16-cell stage were segregated and cultured in small watch glasses for a period corresponding to that required by controls to reach the mesenchyme blastula or early gastrula stage. Analyses of cytochrome oxidase in these isolates indicated that cytochrome oxidase synthesis occurred more rapidly in cells derived from the mesomeres. In five experiments a significantly higher (40 per cent) cytochrome oxidase activity was found in homogenates of animal as compared to vegetal isolates.

The results are not stressed and must be questioned, since the isolated animal and vegetal pairs did not develop into blastulae. Even after thorough washing of the cells with sea water the disassociation process partially continued and subsequent cleavages formed clumps of unorganized cells. Cytochrome oxidase values of these were low as compared to normal blastulae of equivalent age which suggests a lagging of enzyme synthesis.

These uncertainties thus do not permit any conclusion as regards development of a cytochrome oxidase gradient in normal later stages of *Dendraster*. In order to avoid these difficulties attempts were made to cut gastrulae in

alves with a microneedle with subsequent determination of cytochrome xidase in isolated halves. It was not possible, however, to obtain sufficient aterial for analyses by this procedure.

SUMMARY

Animal and vegetal cells of the 16-cell stage of the sand dollar, *Dendraster*, ere disassociated and segregated in sufficient quantities for microchemical nalyses of cytochrome oxidase. No significant difference in cytochrome xidase activities was found in homogenates of these cells. Continued eavage of animal and vegetal isolates led to unorganized masses of cells in hich cytochrome oxidase activity was higher in derivatives of the mesomeres.

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PHYSIOLOGICAL STUDIES OF DIFFERENTIATION IN *MYTILUS EDULIS*

II. ACCUMULATION OF PHOSPHATE IN ISOLATED BLASTOMERES AND POLAR LOBES

W. E. BERG and D. M. PRESCOTT

Department of Zoology, University of California, Berkeley, Calif., U.S.A.

Received August 15, 1957

An early determination and segregation of developmental factors occurs during the development of *Mytilus edulis* [14] similar to that which occurs in other mollusks [15]. Methods have been developed for separation and segregation of blastomeres and polar lobes in sufficient numbers to permit quantitative biochemical and physiological studies of the different cytoplasms [3]. Studies of the oxidative metabolism [5] and quantitative measurements of peptidases [4] have been carried out on isolated blastomeres and polar lobes. The present study, representing a continuation of attempts to characterize the different cytoplasms, was undertaken to see if physiological differences of isolated blastomeres and polar lobes might be reflected by different rates of phosphate accumulation.

METHODS

Eggs and sperm were obtained from *Mytilus edulis* by allowing the animals to spawn in individual dishes of sea water. Egg membranes were removed with a membrane lysin extracted from sperm [2] and the first cleavage blastomeres were separated in Ca-free sea water. The blastomeres were transferred to Ca-free sea water containing P^{32} as orthophosphate (5–10 μc/ml). Shortly before the next cleavage the blastomeres were removed, washed thoroughly by transference through five or more dishes of sea water and segregated with a braking pipette. Reliable measurements of radioactivity could be obtained on fifty CD blastomeres and one hundred AB cells.

A similar technique was used for measuring phosphate uptake of C and D blastomeres. Isolated CD blastomeres were allowed to cleave in Ca-free sea water, and the separated C and D cells were treated as described above for isolated first-cleavage blastomeres.

The phosphate accumulation by polar lobes was compared to that of AB blastomeres. Polar lobes were constricted from eggs by hypertonic salt solutions and in some experiments segregated by the elutriator [3]. In each experiment the polar lobes and AB blastomeres were exposed to the same radiophosphate solution for identical intervals.

Volumes and surface areas of the blastomeres and polar lobes were calculated rom average diameters as measured with an ocular micrometer. The surface areas s calculated are undoubtedly less than the effective uptake areas. Electron micro- raphs show the presence of microvilli at the surface of *Mytilus* eggs; however to hat extent these increase the surface area could not be determined. For the purposes f comparison it was necessary to use the calculated values.

RESULTS

The uptake of phosphate, using the procedure outlined above, was deter- ined for AB, CD, C and D blastomeres and polar lobes. Since only relative ates of uptake were desired, the results were calculated as counts per minute er volume and surface area.

The first cleavage of the *Mytilus* egg is such that the CD blastomere is twice ie volume of the AB cell. Cleavage of the CD blastomere is also unequal esulting in a 2:1 volume ratio of the D and C cells. Volumes of isolated olar lobes are more variable; however, the average volume is one-third hat of the AB cell. Usually in each experiment the number of cells chosen as such that radioactivity measurements were made on equal volumes of ytoplasm. Thus the phosphate uptake of 100 AB blastomeres was compared ith 50 CD cells, 75 D cells compared to 150 C cells and 200 polar lobes ith 66 AB cells.

Table I summarizes the data obtained on phosphate uptake of the various ells. For brevity the results are expressed as average ratios of uptake per olume and surface area as obtained from a number of paired experiments.

The CD blastomere accumulates, within the same length of time, 81 er cent as much phosphate per unit volume as the AB blastomere. The D lastomere likewise accumulates phosphate per unit volume at a slower ite than the C cell. An even more striking difference is obtained by comparing ^{32} uptake per unit volume of polar lobes with AB blastomeres. All of these ifferences are statistically significant.

ABLE I. *Relative rates of radiophosphate accumulation in isolated blastomeres and polar lobes of* Mytilus edulis.

Blastomeres	Number of experiments	Ratio of accumulation rates of P^{32} per unit volume cytoplasm	Ratio of accumulation rates of P^{32} per unit surface area
D/AB	12	0.81 ± 0.02	1.02 ± 0.02
/C	10	0.65 ± 0.04	0.83 ± 0.05
olar lobes/AB	5	0.66 ± 0.02	0.48 ± 0.02

Since, as will be indicated later, the limiting factor in phosphate uptake may be a surface phenomenon, the results have also been calculated in terms of surface areas of the cells. If the accumulation of phosphate of AB and CD blastomeres is expressed as uptake per unit surface area there is no significant difference between the cells. However, a similar comparison between C and D blastomeres indicates that the P^{32} accumulation per unit surface area is significantly greater for the C blastomeres. Likewise the uptake of AB blastomeres per unit surface area is twice that of polar lobes.

Accordingly, whether phosphate accumulation is expressed in terms of volume or surface area there is a significant difference between the C and D blastomeres and an even greater difference between AB cells and polar lobes.

It has been shown that in the sea urchin egg over 95 per cent of the labeled phosphate is incorporated into the labile (acid soluble) compounds and the remaining small amount into the nonlabile (acid insoluble) substances [8, 1]. The *Mytilus* egg is similar in that most of the labeled phosphate is incorporated into the acid soluble constituents.

Fertilized *Mytilus* eggs were allowed to take up labeled phosphate for thirty minutes. These were washed and the radioactivity measured. After extraction with 5 per cent trichloracetic acid the activity was again determined. The average of five experiments gave a value of 96 per cent of the labeled phosphate in the acid soluble fraction and 4 per cent in the acid insoluble fraction.

It was of interest to see whether a difference in phosphate accumulation existed between blastomeres if only the acid insoluble fraction was compared. Accordingly AB and CD blastomeres, after accumulating labeled phosphate, were washed with trichloracetic acid. The remaining radioactivity, representing phosphate in the nonlabile compounds, was measured in 300 CD and 600 AB blastomeres for each experiment. In six experiments the average value of phosphate accumulation per unit volume by CD cells was 25 per cent less than that of the AB cells. Thus the difference in total phosphate accumulation of blastomeres (Table I) is reflected by a similar difference in accumulation within the nonlabile fraction.

Several other experiments were carried out, the results of which will be reported briefly.

During cleavage of the *Mytilus* egg the cytoplasm at the vegetal pole (polar lobe) becomes incorporated into the CD blastomere. The data in Table I suggests that this cytoplasm incorporates phosphate at a slower rate than the cytoplasm entering the AB cell. It was of interest to determine whether

is polar lobe cytoplasm incorporated phosphate at a slower rate while still part of the whole egg.

Eggs, a few minutes after fertilization, were allowed to take up phosphate ntil just prior to cleavage. A few minutes before cleavage was expected ey were removed, washed and allowed to cleave in Ca-free sea water. ne blastomeres were separated and the radioactivity of each type deter- ined. The data from nine experiments indicated no significant difference the amount of labeled phosphate in the AB and CD cells after correction r volume differences.

In the above experiment the total labeled phosphate was measured, most which was contained in the labile compounds. It seemed possible that gional differences of phosphate accumulation within the whole egg might demonstrated if only the more stable nonlabile compounds were con- dered. Accordingly the above experiment was repeated; however the astomeres were given a final washing in trichloracetic acid before meas- ing radioactivity. Again no significant differences were found between the nount of labeled phosphate in the AB and CD blastomeres after correction r volume differences.

It therefore seems that in the egg prior to cleavage the cytoplasm destined form the CD cell accumulates phosphate into both the labile and nonlabile actions to the same extent as that forming the AB cell.

DISCUSSION

The rapid phosphate uptake by *Mytilus* eggs is undoubtedly by a process accumulation as in the sea urchin egg [7, 9]. In sea urchin eggs phosphate, aying a key role in cellular metabolism enters a wide variety of compounds thin the egg and it is presumed to do likewise in the *Mytilus* egg. A frac- nation of the labeled phosphate in *Mytilus* eggs indicates that 96 per cent ters the labile compounds and the remaining enters the nonlabile com- unds.

The nature of the limiting factors in the phosphate accumulation process not clear. In general phosphate uptake seems to be closely related to cel- ar activity and is greater in actively metabolizing cells. Fertilization of the urchin egg is followed by radical metabolic changes and is accompanied a marked increased phosphate uptake. The phosphate accumulation en shows a close correlation with oxidative metabolism and Chambers d White [9] are inclined to the view that accumulation of phosphate may explained by "driving forces" in the cell, i.e., rates at which phosphate

undergoes esterification within the cell. This would imply that phosphate accumulation might be directly related to metabolic activities within the cell. Additional support for this is the observation that slight variations in oxidative metabolism during cleavages of the egg are duplicated by variations in phosphate uptake [16]. However, a lack of correlation occurs in the *Chaetopterus* egg where a post-fertilization drop in oxygen consumption is accompanied by a rise in phosphate uptake (Berg, unpublished data).

It has been suggested that penetration of phosphate into a cell requires esterification at the cell surface [11, 12], and it is conceivable that this process could be a limiting factor in phosphate accumulation. Lindberg [12] concludes that phosphate is incorporated into adenosinetriphosphate by a phosphorylation mechanism at the cell surface and that the rate of transportation of ATP to the interior of the cell is a limiting factor for the entire process.

The observation that phosphate uptake of AB and CD blastomeres is directly related to surface area indicates that the limiting factor may be one of surface activity rather than internal cellular metabolism. The failure to demonstrate regional differences of phosphate accumulation into either the labile or nonlabile fractions of the egg prior to cleavage also argues against interpreting the data as reflecting differences in internal metabolic activities.

If a surface activity such as rate of phosphorylation or diffusion of ATP is limiting then it must be concluded that a change in this factor occurs during cleavage of the CD blastomeres whereby the D blastomere has a lower rate of phosphorylation. The low phosphate uptake rate of isolated polar lobes suggests that the surface of the polar lobe cytoplasm, normally a part of the D cell, may be responsible.

The presence of microvilli, as revealed by electron micrographs, at the surface of *Mytilus* eggs must greatly increase the surface area effective in phosphate uptake. Changes in appearance of the microvilli have been observed in the vegetal region of the egg [10] and it is possible that such changes might alter rates of uptake of phosphate.

It is conceivable that a low phosphate uptake of polar lobes is due to a lack of nuclear material as it is known that enucleated amoebae have a reduced phosphate uptake [13, 6]. However, enucleated amoebae show a rapid and continual decrease in phosphate uptake [6] whereas the rate of accumulation of phosphate by polar lobes remains constant and only undergoes a decrease after 24 hours. Furthermore, it is evident that the difference in phosphate accumulation of C and D blastomeres cannot be caused by lack of nuclear material.

Although there may be no direct correlation it should be pointed out that these data parallel those obtained on oxygen consumption of isolated blastomeres and polar lobes of *Mytilus* [5].

SUMMARY

Accumulation rates of radioactive phosphate were determined for eggs, isolated blastomeres, and polar lobes of *Mytilus edulis*. A fractionation of the labeled phosphate in eggs indicates that 96 per cent enters the labile compounds and the remaining enters nonlabile compounds. No regional differences in uptake rates of phosphate into either labile or nonlabile compounds could be demonstrated in the fertilized egg prior to cleavage. After cleavage the uptake of phosphate into the AB and CD blastomeres correlates with surface area. Isolated polar lobes and D blastomeres, however, exhibit a significantly lower accumulation rate of phosphate than AB and C blastomeres. Limiting factors in phosphate uptake are considered and it is possible that the differences in accumulation rates may reflect changes at the cell surface.

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THE LOCALIZATION OF INORGANIC PHOSPHATE IN THE OÖCYTES OF *BUFO ARENARUM* HETEROGENEITY OF THE NUCLEOLI

C. J. TANDLER

Instituto Fitotécnico de Santa Catalina, Llavallol, Argentina

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Inorganic phosphate has been shown to be a normal component of the nucleolus; the nucleolar phosphate, like the nucleolus itself, undergoes cyclic disappearance and reappearance at mitosis [14].

Amphibian oöcytes have multiple nucleoli of different sizes and staining characteristics; extrusion of nucleolar materials through the nuclear membrane into the cytoplasm and its possible role in protein synthesis have been reported [4, 7, 10, 15]. In the present paper the localization of inorganic phosphate in the oöcytes of *Bufo arenarum* was investigated.

EXPERIMENTAL METHODS AND RESULTS

Small portions of the ovary were obtained from toads killed by destruction of the spinal cord and fixed without delay. The tissue was dehydrated in ethanol, cleared in cedar wood oil and imbedded in paraffin; sections were cut at 6–8 μ. The degree of shrinkage, although dependent on the kind of fixatives used, is minimized by the cedar oil which does not make the oöcytes brittle. The following procedures have been employed.

The lead acetate reaction for inorganic phosphate. The tissue was fixed in 5 per cent neutral lead acetate at room temperature for 12 hours, hardened

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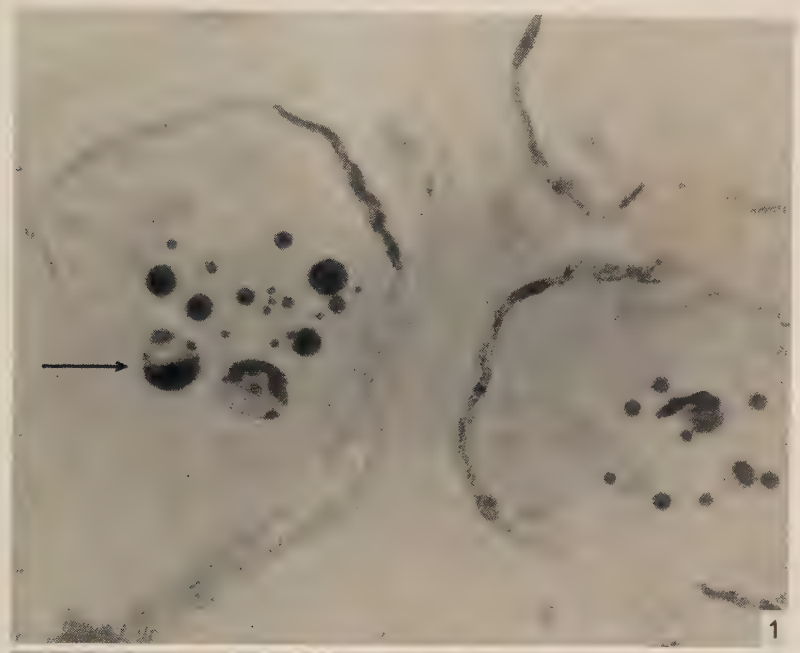
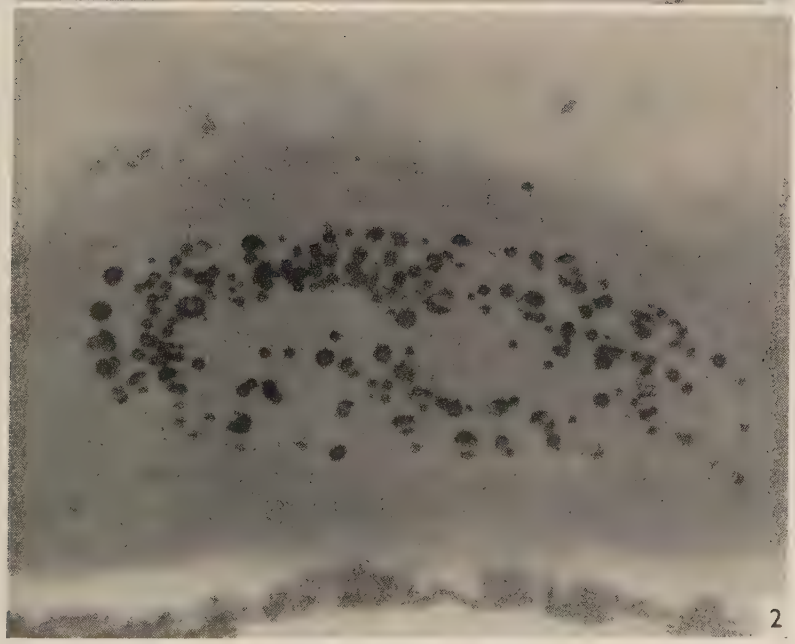
Sections through the ovary of *Bufo arenarum*. Fixation, 5 per cent lead acetate. Staining reagent, H_2S. No counterstain was employed.

Fig. 1.—Two oöcytes approximately 70 μ in diameter. Note that only a portion of the large spherical nucleolus 10 μ in diameter (indicated by the arrow) gives a positive reaction for inorganic phosphate. Other nucleoli can be seen within the same nucleus measuring approximately 43 μ in diameter. The nucleoli of the small follicular cells also react positively.

Fig. 2.—More than hundred nucleoli within a nucleus measuring 114 × 34 μ give a positive reaction for inorganic phosphate. Note that some nucleoli are formed by lead acetate-negative and lead acetate-positive portions. Light cytoplasmic stain is non-specific and occurs also in trichloroacetic acid-treated control sections.


1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in a 5 per cent lead acetate solution in 10 per cent formalin and imbedded in paraffin. The sections were treated as described previously [14]. Inorganic phosphate was found to be localized only in the nucleoli; the cytoplasm of the oöcytes does not react at all (Figs. 1 and 2). When the sections are directly stained with H_2S, omitting all the intermediate steps, the yolk platelets stain brown and the nucleoli black. Washing in distilled water does not suppress the binding of lead to the platelets; treatment with lead acetate in glacial acetic acid [14] followed by distilled water eliminates almost completely the staining of Pb-yolk proteins without affecting the reactivity of the nucleoli. Immersion in cold 5 per cent trichloroacetic acid eliminates the nucleolar reaction. Nucleoli of different sizes react similarly (Fig. 1). The smaller nucleoli of the follicular cells also react positively. It is known that the nucleoli frequently appear as a non-homogeneous body formed by two clearly differentiated (basophil and oxyphil) portions [7, 10, 15]. We found the nucleoli to be differentiated into lead acetate-negative and lead acetate-positive portions (Fig. 1). Comparing the Pb-sections to others stained with toluidine blue it appears that the inorganic phosphate is localized in the basophil portion (basophilia attributable to ribonucleic acid [1]).

Argentaffin reaction.—It has been shown that the nucleolus reduces ammoniacal silver nitrate in darkness [12]. The tissue was fixed in 10 per cent formalin, washed with running tap water and stained as described previously [12]. The silver reagent was used at 45–55°C for two to 6 hours and observations were made every hour. The nucleoli were found to reduce ammoniacal silver nitrate more rapidly and strongly than other cell parts (e.g. yolk platelets, nuclear sap) and to be clearly differentiated into negative and positive argentaffin portions.

Other cytochemical procedures.—The tissue was fixed in alcohol–formalin–acetic acid (85:10:5), Gendre's [9] and Champy's fluid. Excellent preparations have been obtained with Champy's fixative. The nucleoli of the oöcyte germinal vesicle do not respond to the Feulgen, plasmal, periodic acid– or lead tetraacetate–Schiff techniques. Glycogen is present in appreciable amount between the yolk platelets and as a perinuclear ring; it appears to be the only periodic acid–Schiff positive substance in the cytoplasm, as all the reaction is prevented by prior saliva or diastase digestion [9]. After being stained with Sudan black (frozen sections of gelatin-imbedded, formalin-fixed tissue) the oöcyte show numerous small lipid droplets between the yolk platelets, which sometimes fuse artefactually into larger droplets. The vitelline membrane is probably a mucoprotein or a neutral mucopolysaccharide; it gives a strongly positive periodic acid - and lead tetraacetate–

rchiff reactions which are not removed by saliva, it does not stain meta-hromatically with toluidine blue and is Millon and ninhydrine-Schiff [2] iositive.

DISCUSSION

In 1898 Montgomery [11] stated that "the true nucleolar substance probably as no chemical relation to the true chromatin (nuclein)". The intimate ssociation between nucleoli and chromosomes raised the hypothesis that he nucleoli were the source of the chromatin material. With the recognition hat the Feulgen reagent was specific for desoxyribonucleic acid this possi-ility became less likely, and the nucleolus began to figure more prominently is involved in nuclear-cytoplasmic interchanges [15]. Although a "physio-pgy" of the nucleolus has yet to be developed, it is significant that the pplication of new cytochemical techniques showed that some substances eemed to be specifically located in the nucleoli. The argentaffin and the norganic phosphate components are two essentially different substances [13] lthough both are located in the nucleoli. In the case of the amphibian egg ells the germinal vesicle has hypertrophied multiple nucleoli; only the baso-hil portion of these nucleoli gives positive lead acetate and argentaffin eactions. There is no doubt that the PbS stain reflects the localization of a ead phosphate precipitate and not merely an "affinity" for lead (see [14]). t might be questioned whether or not the nucleolar lead phosphate truly epresents the *in vivo* localization of inorganic phosphate. One evidence gainst diffusion of phosphate into the nucleoli during lead acetate fixation s the known fact that diffusion artefacts also stain the chromosomes; no recipitate can be detected on the chromosomes after lead acetate fixation nd *nothing* is stained in a cell at metaphase [13, 14]. It remains to be an-wered how much of the *total* inorganic phosphate of the whole cell is present precipitated as lead phosphate) in the nucleolus. Fixation in alcoholic Pb xatives (or treatment with lead acetate after alcohol fixation) yields negative nages; it is probable, then, that inorganic phosphate is not present in the ucleoli as an insoluble salt (e.g. calcium phosphate). It was suggested that hosphate is adsorbed or bound in an unknown manner to some other omponent of the nucleolus [14]; this hypothesis proved to be necessary since ison [9] established that it is impossible to localize diffusible substances of ow molecular weight by precipitation with salts of heavy metals. A number f authors, based on observations of fixed material, have described extrusions f nucleoli or nucleolar materials through the nuclear membrane into the ytoplasm [7, 15]. We have not found any indication of lead acetate positive

materials in the cytoplasm, so that if some substance is transferred to the cytoplasm it probably comes from the lead acetate negative portions. We have seen nucleoli in the cytoplasm or even halfway through the nuclear membrane, but, as other authors have pointed out [6, 8], they have been simply dragged to this position by the microtome blade; it was never found when sectioning is omitted (staining the tissue in the block).

A peculiarity of the nucleoli at certain stage of the oögenesis corresponding to Duryee stage 3 [4] ought to be mentioned. At this stage the nucleoli lie peripherically against the nuclear membrane; it was found that it is the oxyphil portion which attaches to the membrane while the basophil portion looks towards the center of the germinal vesicle (fixation in alcohol–formalin–acetic acid). Cell measurements indicate that the diameter of the oöcytes is between 200 and 300 μ; in smaller or larger oocytes the phenomena is not so evident. This recalls Ludford's hypothesis [10] that the oxyphil portion of the nucleoli extrudes portions of itself into the cytoplasm. Gall [6] observed that the peripherally located nucleoli are quite firmly attached to the nuclear membrane: when the membrane is removed from one of the small eggs the chromosomes float free but the nucleoli do not. In older eggs the nucleoli are free in the nucleoplasm. These observations support the thesis of nucleolar-cytoplasmic interchanges. According to Estable and Sotelo [3, 5] the "nucleolonema" constitutes a permanent nucleolar structure associated with the chromosomes; during interphase, the nucleolus accumulates other materials, the *pars amorpha*. The argentaffin substance and the inorganic phosphate must be classed as components of the *pars amorpha* since they disappear completely during mitosis and no reaction at all can be detected on the chromosomes.

SUMMARY

Inorganic phosphate has been localized in the nucleoli of the oöcytes of *Bufo arenarum*. The nucleoli are formed by two clearly differentiated (basophil and oxyphil) portions but only one of them contains inorganic phosphate and gives a positive argentaffin reaction.

I wish to acknowledge the interest of Professor J. M. De Carlo in this work.

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MYELIN SHEATH STRUCTURE IN NERVE FIBRE DEMYELINIZATION AND BRANCHING REGIONS[1]

H. ENGSTRÖM and J. WERSÄLL

Department of Otology, University of Göteborg, and Department of Histology and Anatomy, Karolinska Institutet, Stockholm, Sweden

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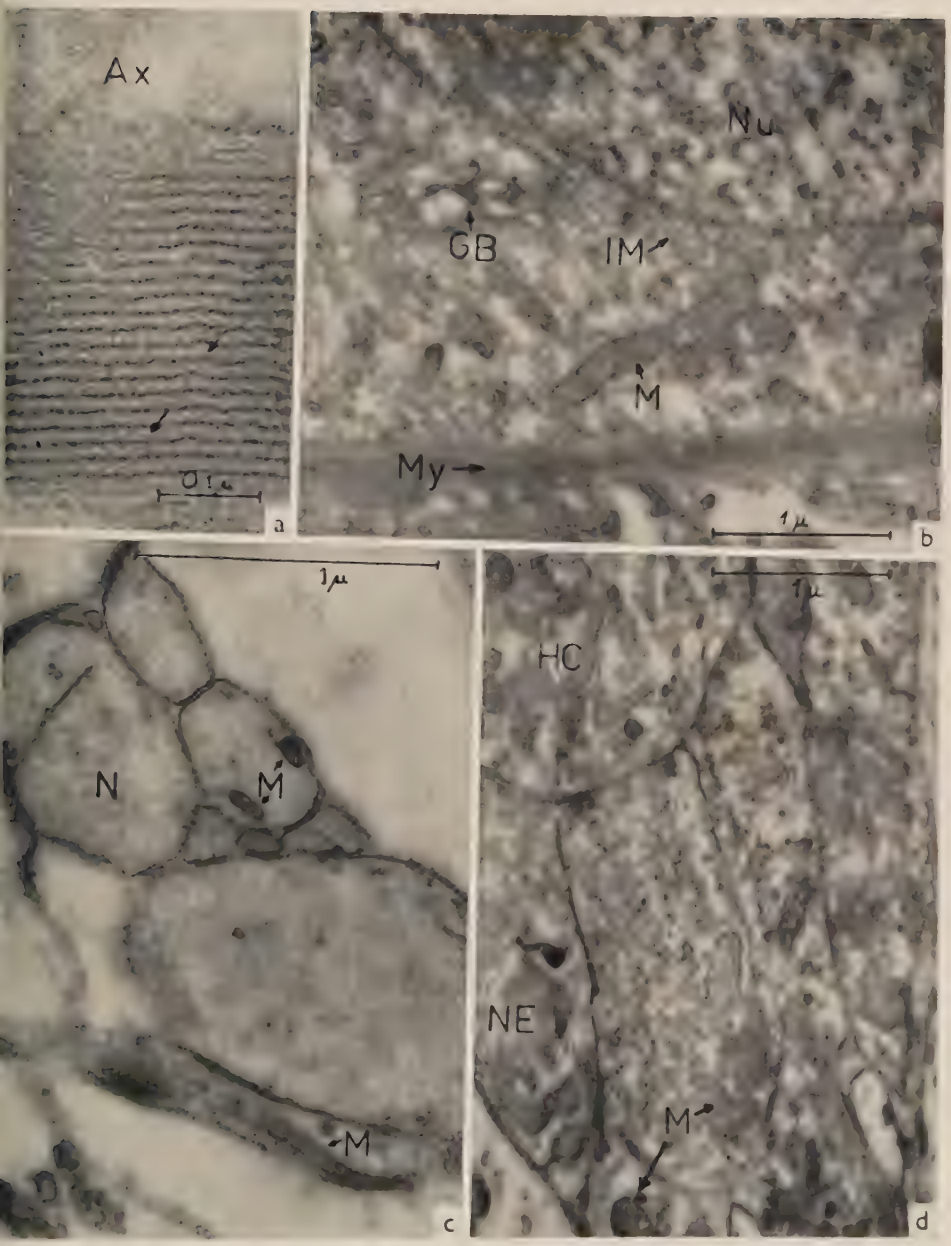
Many authors have carefully described the structure of the myelin sheath in the internode portion of peripheral nerves. The use of polarized light, X-ray diffraction and especially the electron microscope have now enabled us to analyze the very regular and characteristic arrangement of the myelin sheath. Fernández-Morán [8], a pioneer in the study of submicroscopic nerve fibre structure, recently published a comprehensive review of our present knowledge of the nerve.

However, in all the literature available in this field very little is said about the structure of the myelin sheath at Ranvier's constrictions or nodes and even less about the manner in which the nerve fibre sheds its myelin sheath peripherally.

The various sensory epithelia of the inner ear and the taste buds contain numerous nerve fibres which end in a restricted area and shed their myelin sheaths in the immediate neighborhood of the basement membrane of the epithelium. In the course of previous studies of these ephithelia we have assembled a comprehensive series of sections in which peripheral nerve fibres are undergoing demyelinization. Considering that the structure of the myelin sheath appeared to be very complex in the region of demyelinization, we attempted to analyze the ultrastructure of the myelin sheath in the region of the peripheral nerve fibre demyelinization. The results of the analysis are presented in the present paper.

[1] This report has been supported by grants from the Swedish Medical Research Council.

Fig. 1.—Cross sectioned myelin sheath from the acoustic nerve with regularly arranged lipid-protein layers. The arrows indicate intermediate lines; *B*, micrograph from a spiral ganglion cell with myelin coating (*MY*), mitochondria (*M*), Golgi body (*GB*) and nucleus (*Nu*); *C*, unmyelinated nerve fibres in the organ of Corti (*N*) containing elongated mitochondria (*M*); and *D*, nerve endings (*NE*) below the base of an outer hair cell (*HC*). The richly granulated variety of the nerve endings are very large and have an extra double membrane inside the hair cell plasma membrane. At the 1 μ sign a small nerve ending can be seen without any such intracellular membrane. The nerve endings contain clusters of mitochondria.


Ax
0.1 μ
a
Nu
GB
IM
M
My
1 μ
b
1 μ
N
M
M
c
1 μ
HC
NE
M
d

MATERIALS AND METHODS

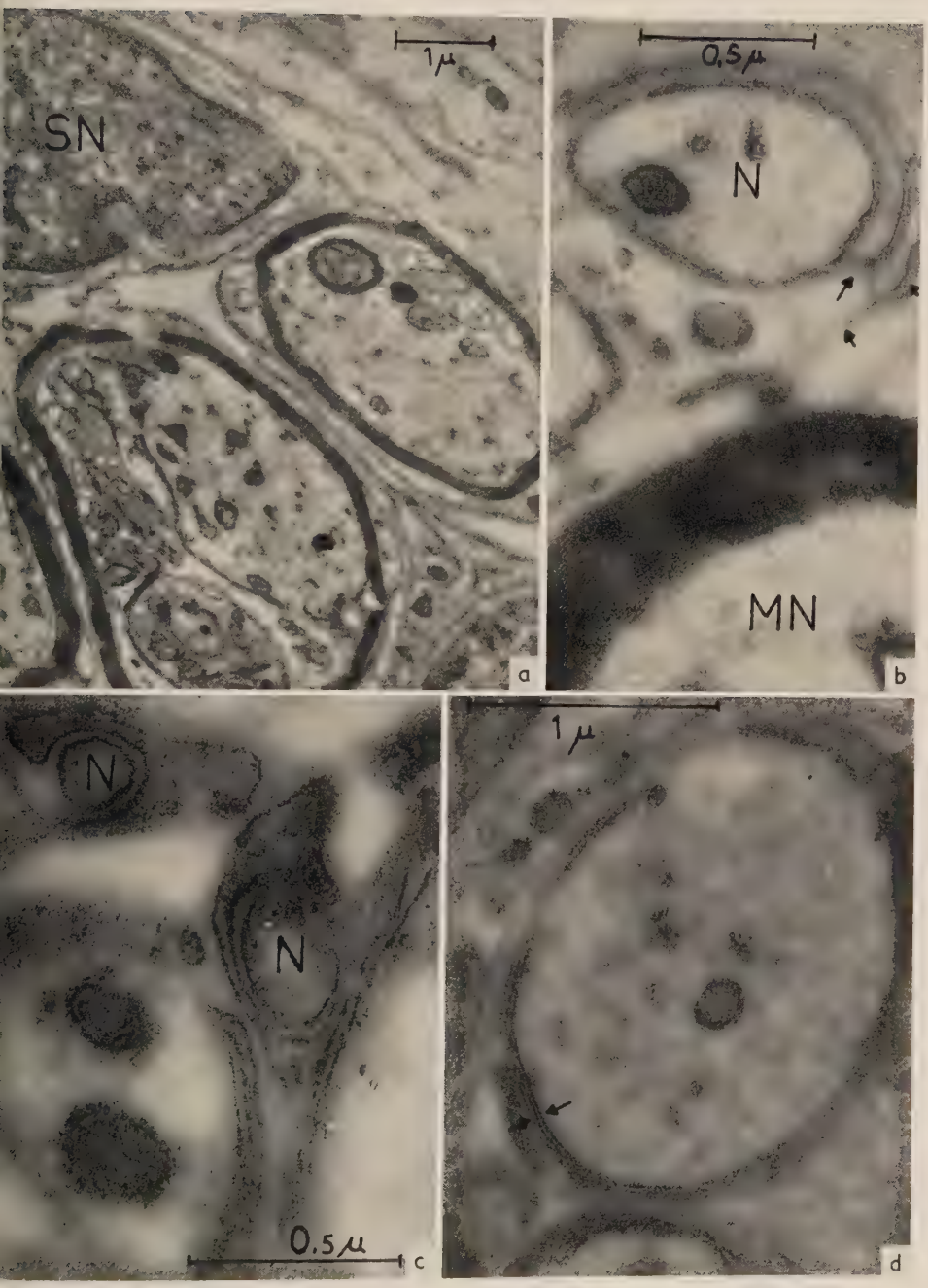
The material consisted of a series of rabbit foliate papillae with underlying nerves and a large number of inner ears from guinea-pig prepared for studies of the membranous labyrinth and its innervation (cf. [1, 2, 3, 4]). The latter material in particular is extremely suitable for analyzing the demyelinization process. Some twenty or thirty thousand nerve fibres shed their myelin sheaths in the well defined and easily recognized region of the osseous spiral lamina of the cochlea, and consecutive serial sections of adequate size can be produced from this region.

Our specimens were fixed in veronal-buffered osmium tetroxide solution [12], rendered blood-isotonic according to Sjöstrand [16] or in a 1 per cent osmium tetroxide solution containing 3 per cent dextran, 0.5 per cent NaCl and 0.2 per cent sucrose. The specimens were sectioned with diamond [7] or glass knives on a microtome of Sjöstrand's design, the sections being photographed in an RCA model EMU 3 C electron microscope.

THE MYELINATED NERVE AND ITS DEMYELINIZATION

According to Schmidt's classical description, the internode portion of the myelin sheath consists of concentrically arranged layers of proteins and lipids. While the present paper was in press, Geren-Uzman and coworkers [11] published a careful description of the ultrastructure of the internode portion of the myelinated nerves. These authors observed many of the organizational principles we have described with respect to the peripheral end of the myelin sheath. In the light of their observations, our findings indicate that the organization of the myelin as described in the following account is more universally applicable. According to Schmitt, Bear and Clark [15], among others, myelin is a smectic, mixed fluid-crystalline substance with a characteristic and regular pattern. Fundamental electron microscopic studies by Fernández-Morán [5, 6, 7, 8], Sjöstrand [16] and others have disclosed that the myelin sheath exhibits an extremely regular arrangement of concentric layers composed of more or less dense matter. Although details of the arrangement and dimensions of these layers can be found in several publications, their important bearings on the subsequent discussion in this paper makes it justified to repeat the essentials of the arrangement of these layers.

Fig. 2.—*A*, myelinated nerves at the point of division with myelin layers in their interior. In the upper left corner a Schwann nucleus (*SN*); *B*, myelinated nerve (*MN*) and an unmyelinated fibre, where the spiral arrangement of the Schwann protoplasm is very distinct (vestibular epithelium); *C*, unmyelinated nerves (*N*) below the base of a taste bud, partially covered by Schwann protoplasm; and *D*, nerve fibre from a taste bud region with three opaque layers (arrow) formed by the Schwann cell.


1μ
SN
a
0.5μ
N
MN
b
N
N
0.5μ
c
1μ
d

When a myelinated nerve is examined in cross section, the myelin sheath appears to be composed of alternating opaque and less opaque layers arranged concentrically around the axon. The opaque layers are some thirty or forty Å thick. Occasionally these are divided into two thinner zones separated by a less opaque zone, all zones having approximately the same thickness. The opaque layers are, according to Geren [9], formed by the fusion of two overlapping layers of the plasma membrane of the surrounding sheath of Schwann which is coiled helically around the nerve fibre axon. This interpretation is verified by Robertson [13], Wersäll [18] and others.

Interposed between two opaque layers lies a less opaque layer having a thickness of 60 to 80 Å. About halfway across this layer there is a narrow, opaque line whose width ranges from 30 Å down to 10 Å or even less. The regular arrangement of the opaque and less opaque layers was clearly visible in our sections. (See the photomicrograph in Fig. 1*A*.) The myelin sheaths of the spiral and vestibular ganglion cells exhibit the same pattern.

The two outermost layers of the myelin sheath have not fused, but are continuous with the external plasma membrane coating of the Schwann cell. Inside the Schwann cell a nucleus, mitochondria and Golgi apparatus are discernible. Cytoplasmic masses from the Schwann cell are occasionally seen encapsulated between the two normally fused portions of the main opaque layers of the myelin sheath proper, particularly of those myelin sheaths which surround the ganglion cells.

When it approaches a sensory epithelium, in general immediatley beneath the epithelial basement membrane, a myelinated nerve usually sheds its myelin sheath. Spoendlin [17] described the structure of the nerve fibres and their mode of shedding their myelin sheaths in the osseous spiral lamina of the guinea pig cochlea. While Spoendlin's observations and our findings admittedly show certain similarities, his analysis of the demyelinisation process is not entirely correct owing to the inadequate resolution of his electron micrographs.

Using high-resolution micrographs, we have studied the myelin sheath in the demyelinization region. By so doing we have gained some new and interesting information regarding not only the demyelinization process but also about the structure of the myelin sheath.

The axoplasm of the nerve fibre is bounded by a distinct membrane, the axolemma, having a thickness of some 30 to 40 Å. This membrane is separated

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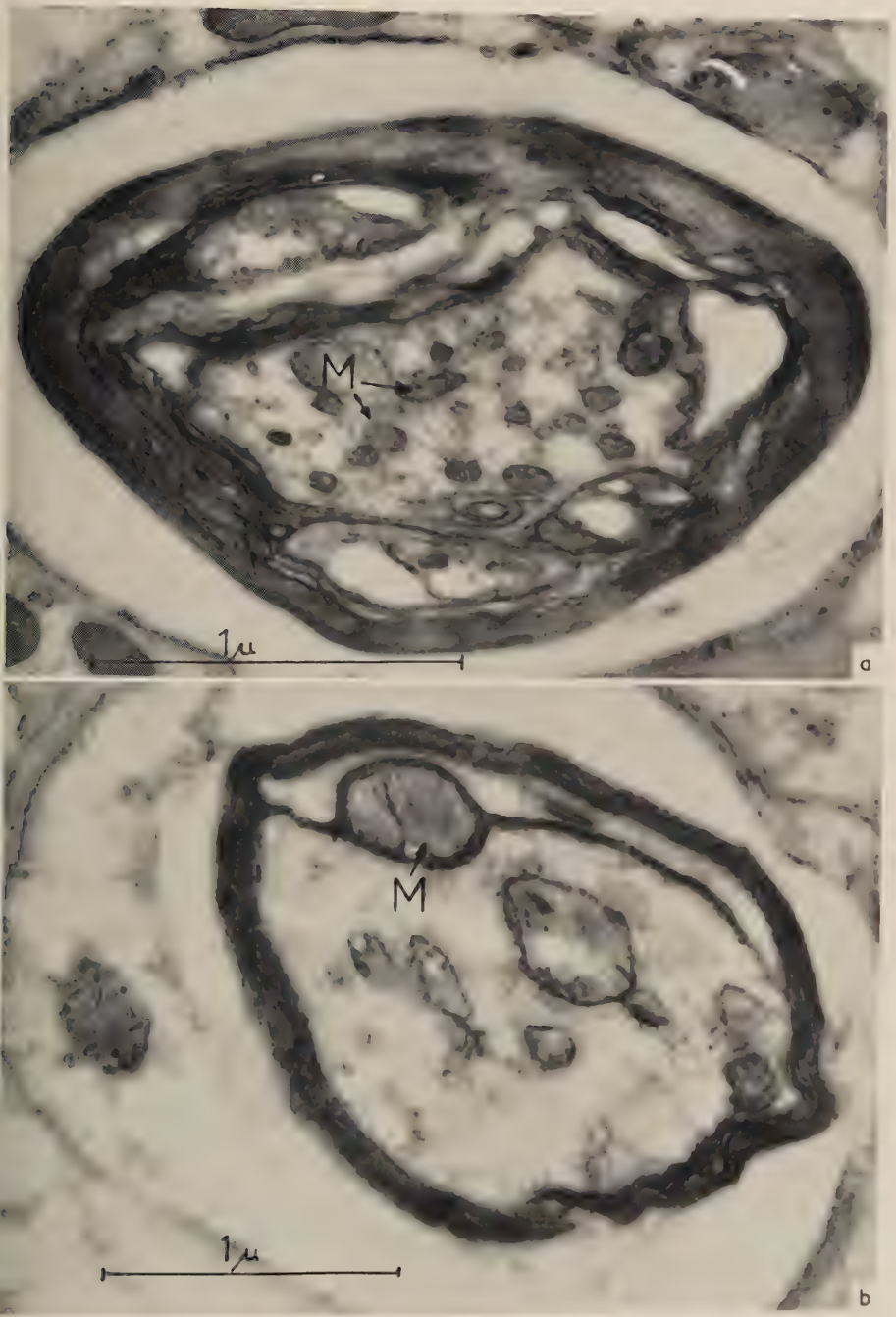
Fig. 3.—Myelinated nerve fibres at regions of division with myelin lamellae in their interior. Mitochondria (*M*).


M
1μ
a
M
1μ
b

from the innermost layer of the myelin sheath by a periaxonic space about 80 Å wide.

As the nerve fibre passes through the region of demyelinization, the number of lamellae in its myelin sheath is progressively reduced in a very characteristic fashion. Thus first the innermost layer and then one by one the outer ones successively uncoil along the central axon. Towards the peripheral limit of each layer of the myelin sheath that has entered the demyelinization region, the two opaque strata of the layer have separated and form a pocket enclosing some Schwann cell protoplasm so that the end of the layer is terminated in a bulging fold (Figs. 4, 6, 7). Thus the appearance of a section passing lengthwise through the nerve is most characteristic in this region: in such a section the terminal "folds" of the myelin layers form a row of swelling bulges disposed along the margins of periaxonic space. (Cf. the simplified drawing in Figs. 6 and 7.)

Accordingly, as the micrographs clearly demonstrate, each of the opaque layers in the myelin sheath is actually a composite of two fused strata. In the adult these strata can only occasionally, for example in the demyelinization region, be distinguished as separate entities. As shown by Geren, however, the two components are readily distinguished during embryonic life.

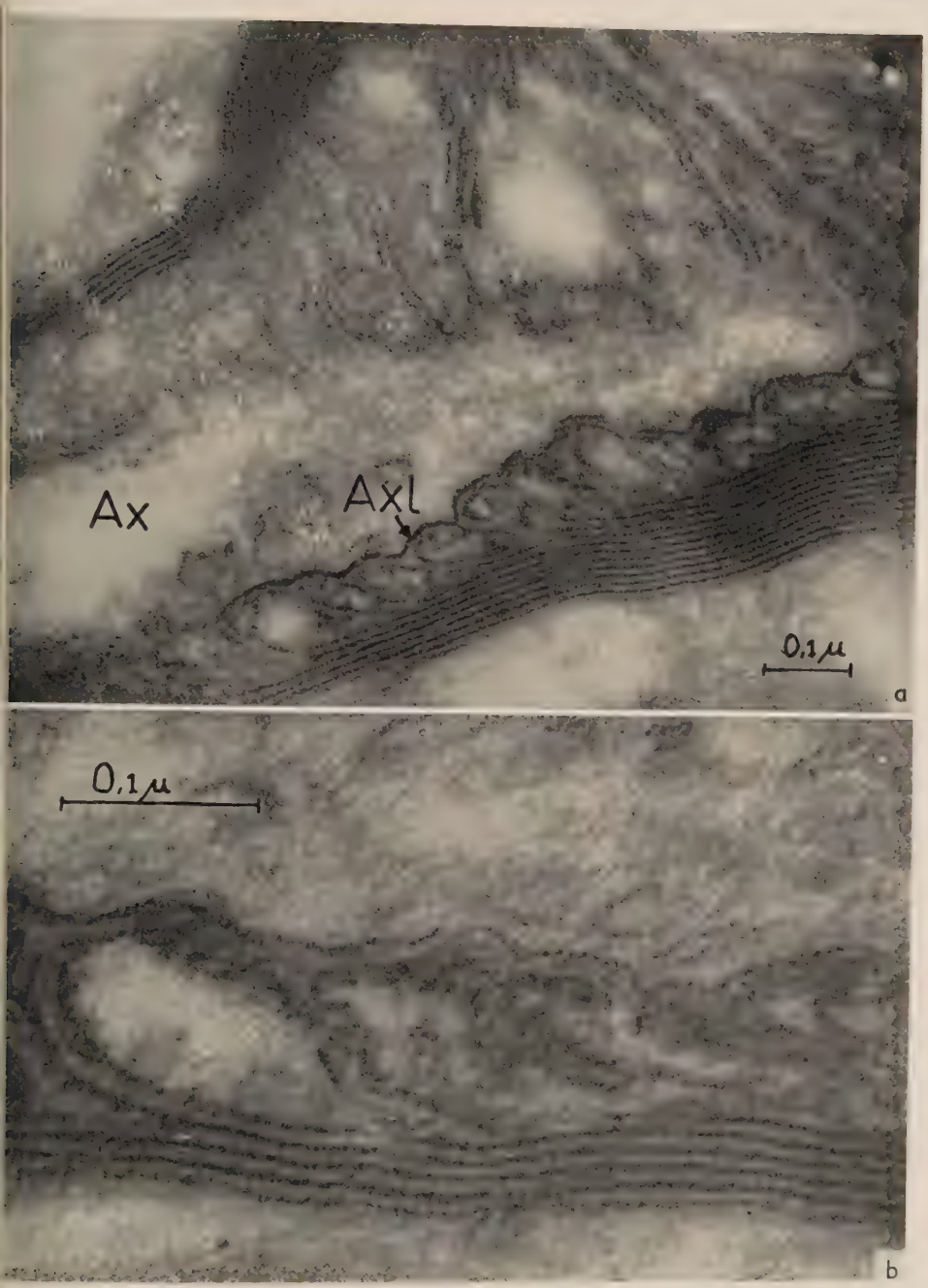
Thus, in the region of demyelinization, the myelin sheath has the appearance of a multilayered ribbon spiraling around the axolemma, the layers becoming progressively fewer with doubly overlapping turns the farther peripherally we look at the nerve. The composite nature of the layers is observable only in the bulging end pouches; farther centrally the two adjacent layers have fused into one.

The myelin sheath's outermost opaque layer represents, as pointed out in the foregoing, only one of a pair of unfused components and is continuous with the plasma membrane investing the surface of the Schwann cell.

The spiral disposition of alternating dark and light layers can readily be discerned in a cross section through a myelinated nerve (Fig. 5).

The appearance of the axon and myelin sheath in a region where a myelinated nerve branches differs somewhat from that seen in other portions of the nerve. In such a region, then, an accumulation of mitochondria and granules can usually be observed in the axon and there is a multiplicity of irregular formations in the myelin sheath. The axonic granules vary from

Fig. 4.—Myelinated fibres at the point where they shed their myelin sheath. The axon (*Ax*) has a distinct axolemma (*Axl*). For a further explanation compare the shematic drawing in Fig. 7.


Ax
Axl
0,1 μ
a
0,1 μ
b

200 to 500 Å in diameter and resemble the synaptic granules occurring in the protoplasm of a variety of different synaptic nerve endings [Fig. 1*D*].

The structure of the myelin sheath investing such parts of the nerve is generally highly irregular. Thus, for example, it is not seldom that myelin lamellae penetrate into the axoplasm and coil themselves into loops around clusters of mitochondria (Fig. 2*A*). These loops also occur disposed as spiral layers around newly formed nerve branches. In sections cut across such parts of the nerve fibres one sometimes finds, as in Fig. 2*A*, myelinated regions within an axon sleeved by another myelin sheath. Apparently the inner myelin sheath of such a pair develops, as do other more irregular myelin formations, from the innermost layer of the regular myelin sheath which—as a pseudopodium from the Schwann cell—continues growing in the axon and thereby gives rise to a separate myelin sheath within the ordinary one.

When it occurs in the most peripheral parts of the fibres innervating the sensory epithelium of the taste buds, where the number of irregular myelin lamellae is comparatively small, such pseudopodial growth around one or more nerve fibre branches by parts of the Schwann cell can be observed particularly well.

As Geren suggested, it seems certain that the opaque layers of the myelin sheath are formed by fusion of two opaque strata derived from Schwann cell plasma membrane. What happens to the cytoplasm originally enclosed between these two strata is unclear but it seems to be compressed in some way and disappears. In the demyelinization region, where the two strata are unattached and form the bulging peripheral endfolds, residues of Schwann cell cytoplasm seem to be present in the pouch thus formed. Next to the plasma membrane lining of the "endfolds" there is an irregular denser zone varying from 50 to 200 Å in thickness. Although in the schematic drawing it is depicted as irregular thickenings of the plasma membrane, the exact structure is not quite clear.

Often the myelin sheath of the vestibular ganglion cell also exhibits a rather irregular disposition, with residues of Schwann cell cytoplasm interposed between the myelin lamellae [19].

The lighter belt separating two opaque myelin sheath layers is not interrupted in the demyelinization region but continues directly into the periaxolemmal space (Fig. 4). Exactly what happens to the opaque line situated intermediately in the lighter belt has not been definitely established, but there

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Fig. 5.—Cross sectioned myelinated nerve (*My*) and axons (*Ax*) covered by a few layers of Schwann protoplasm (*SP*). At the arrows the spiral arrangement of the Schwann protoplasm is very obvious.

Ax
SP
My
M
Ax
1 μ

is some evidence suggesting that it also continues into the periaxolemmal space.

The periaxolemmal space in the fixed specimen often appears uneven or has shallow serrations corresponding to the bulges of the helical endfolds. Whether this is a typical normal feature or an artefact remains to be determined by means of variations of the preparation technique.

After losing its myelin sheath, the axon generally enters the sensory epithelium where it becomes surrounded by sustentacular cells which apparently assume the supporting and isolating functions ascribed to the Schwann cell. Notably, however, in the organ of Corti the axons of the peripheral branches

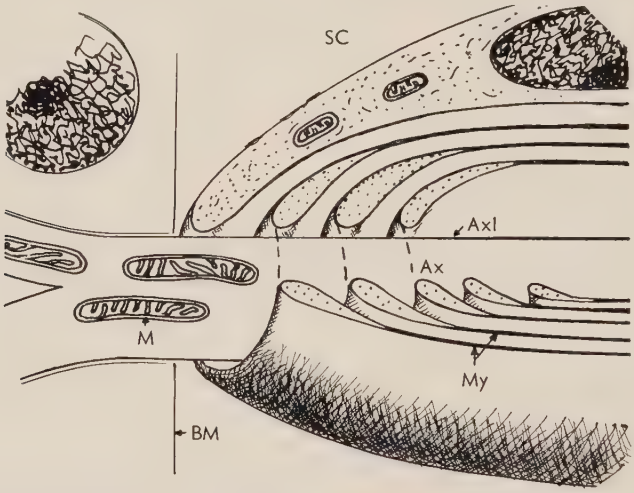

Fig. 6.—Schematic drawing explaining the arrangement of the myelin sheath (*My*) at the region of demyelinization. See Fig. 7.

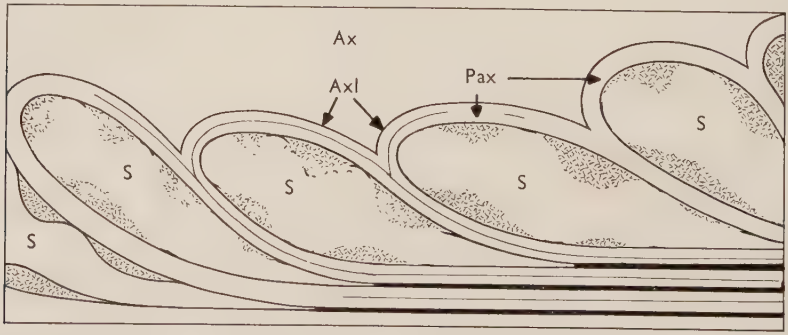

Fig. 7.—Schematic drawing showing the fate of the opaque and less opaque lines of the myelin sheath. Compare with Figs. 4, 5 and 6. *Ax*, axon with mitochondria (*M*). *Axl*, axolemma with periaxolemmal space (*Pax*). The Schwann cell (*SC*) protoplasm (*S*) forms folds.

ay run for a considerable distance stark naked with neither myelin sheath or Schwann cell. Some spironeurons, for example, are unmyelinated over distance of several millimetres. Yet the length of completely denuded xolemma is far from as long. The supporting cells in the organ of Corti rround the nerve fibres and form thin folds around the axolemma. Actually, owever, true multilayered myelin sheaths have never been observed on ich fibres.

The mitochondria in these long naked nerve fibres were often characteristically localized in varicose swellings (Fig. 1 *C*). Fernández-Morán [6] described similar varicose swellings on naked nerve fibres from the spinal ord of the cat.

The peripheral nerve endings were invariably completely denuded and nly a narrow intrasynaptic space separated them from the innervated sensory ells. They contained a profuse accumulation of mitochondria and synaptic anules, as reported by numerous authors previously (c.f. [8]). Notably, owever, similar synaptic granules were also observed in long sections of nmyelinated branches as well as in the most peripheral myelinated fibres, pecially in some of those innervating the sensory epithelia of the vestibular paratus.

SUMMARY

The ultrastructure of the myelin sheath in the demyelinization region of eripheral nerve fibres and their mode of shedding the myelin sheath is escribed in the light of an electron microscopial analysis.

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BRIEF NOTES

THE INHIBITORY EFFECT OF GLUTATHIONE ON SOME PROCESSES OF ANIMALIZATION

S. BÄCKSTRÖM

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

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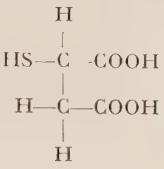
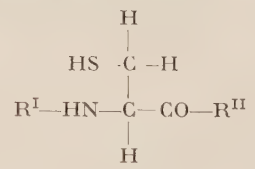
Two antagonistic trends of development, one vegetal and one animal, are involved in early sea urchin development. These trends counteract each other when it comes to determining the entoderm and ectoderm size [8]. By means of some chemical substances, one germ layer or the other may become hypertrophied at the expense of the other [2, 5]. The eggs may thus develop into an animalized blastula or a vegetalized exogastrula instead of into a normal pluteus larva [6, 8]. Animalizing agents are found among many different kinds of substances. Some of the most important of these substances, e.g. SCN ions and *o*-iodosobenzoic acid, are involved in the oxidation of SH groups of the cells [5, 10]. There are also other substances, known to react in some way with SH substances, which bring about animalization and other undefined inhibitions [4, 7, 11]. Thiomalic acid (TMA) is in striking contrast to those substances which react with SH groups. It is a strong animalizer, which is an SH compound itself, and thus constitutes a reducing substance [3]. TMA can easily be oxidized by the specific SH oxidizer, *o*-iodosobenzoic acid (author's experiment).



Thiomalic acid



Cystein moiety of GSH

The similarity between the structular formulae of TMA and the cysteine moiety of glutathione (GSH) suggests that TMA might act as an antimetabolite, i.e. impede the contact of natural GSH with e.g. enzyme protein in certain essential points. If we assume that TMA acts in this way, its action might be counteracted by the addition of GSH.

The following experiment was performed to test the validity of the above presented hypothesis. Soon after fertilization, developing eggs of *Psammechinus miliaris* were treated with m/80–m/160 TMA, or with m/1000 m/2000 GSH, or with both substances simultaneously. Cf. Table I, and Figs. 1–3. The combined treatment with TMA and GSH brought about a much lower degree of animalization than did treatment with TMA alone. GSH also inhibited the formation of cell damage, which is

ABLE I. *Treatment of developing eggs of* Psammechinus miliaris *with thiomalic acid (TMA) and glutathione (GSH).*

rvae counted 48 hours after fertilization in experiment A and 24 hours after in experiment B. The figures indicate the percentage of each class of larvae.

	Experiment A				Experiment B					
	Larvae treated with				Larvae treated with					
	m/160 TMA	m/160 TMA + m/1000 GSH	m/1000 GSH	Normal	m/80 TMA	m/80 TMA + m/2000 GSH	m/80 TMA + m/1000 GSH	m/2000 GSH	m/1000 GSH	Normal
of larvae										
nal blastulae	57	3			84	67	41			
gastrulae	30	9			16	32	57	5	3	5
atic gastrulae	12	15	5	6		1	2	95	97	95
is	1	74	95	94						

ABLE II. *Treatment of developing eggs of* Psammechinus miliaris *with thiomethylcytosine (TMC) and glutathione (GSH).*

rvae counted 48 hours after fertilization in experiment A and 24 hours after in experiment B. The figures indicate the percentage of each class of larvae.

	Experiment A								Experiment B					
	Larvae treated with								Larvae treated with					
	1/4 sat. TMC	1/4 sat. TMC + m/1000 GSH	1/8 sat. TMC	1/8 sat. TMC + m/1000 GSH	1/16 sat. TMC	1/16 sat. TMC + m/1000 GSH	m/1000 GSH	Normal	1/4 sat. TMC	1/4 sat. TMC + m/2000 GSH	1/8 sat. TMC	1/8 sat. TMC + m/2000 GSH	m/2000 GSH	Normal
pes of larvae														
imal blastulae	100	31	94	1					59	37	10	5		
oid gastrulae		69	6	24	12				39	58	47	33	4	5
smatic gastrulae				42	39	6	1		2	5	43	62	96	95
rly plutei				35	42	16	6	6						
utei					7	78	93	94						

ry common in TMA treated material. GSH alone had no morphological effects at in the concentrations here utilized.

An equivalent series of experiments was repeated, now treating the eggs with iomethylcytosine—another animalizing substance, which contains a free SH group one mesomeric form [1]. Also in this case GSH proved to inhibit animalization and l injuries. Cf. Table II, and Fig. 1.

A reaction between equivalent amounts of GSH and SH reagents extinguished the morphogenetic effects of these substances [4]. A similar reaction between GSH and TMA was not to be expected here. Even an imaginary reaction could not explain the inhibiting effect of GSH on the animalizing effect of TMA, as is evident when comparing the concentrations of the two components.

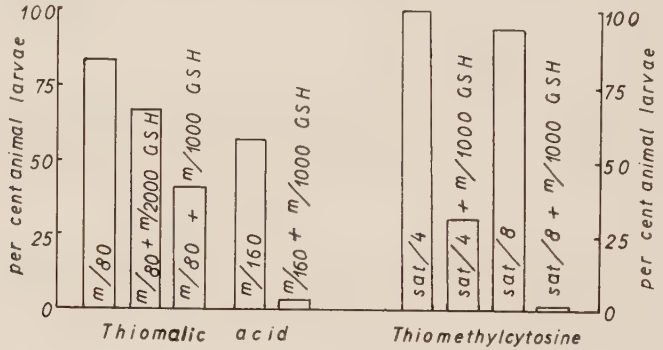
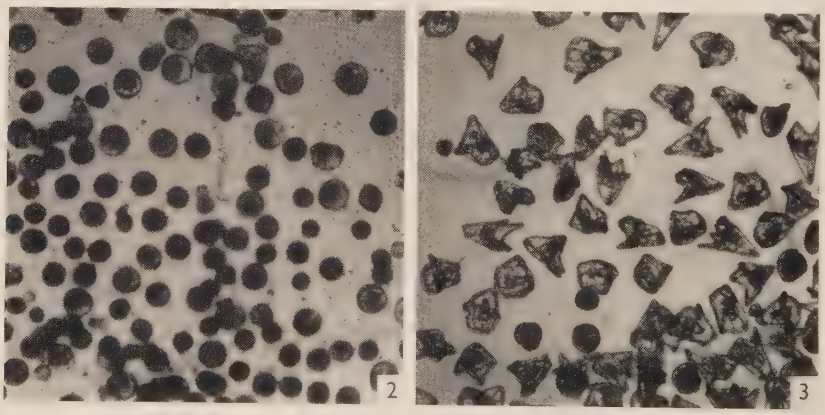


Fig. 1.—Diagram over the share (in per cent) of animalized larvae, obtained after treatment with thiomalic acid (left) and thiomethylcytosine (right), or one of these substances in combination with glutathione.



Figs. 2–3.—Larvae treated with m/160 thiomalic acid (Fig. 2) and with m/160 thiomalic acid in combination with m/1000 gluathione (Fig. 3). Microphotographs taken 40 hours after fertilization with a cine camera on Kodak Super X reversal film.

Morphogenetic effects, provoked by one substance, may be neutralized by another in principally two ways, provided the substances do not react with each other. The two substances may participate either as antimetabolites in the same chain of reaction and compete at a single point, or they may interfere in two different, mutually antagonistic chains of reaction. We can easily find examples from both among the chemical substances, studied with regard to their morphogenetic effects, as is evident by the following comparison.

Li ions, e.g., counteract the animalizing tendencies of thiomethylcytosine, α-oic acid, and SCN [1, 6, 9]. GSH was in this investigation shown to inhibit the ani-alizing tendencies of TMA and of thiomethylcytosine. There is, however, a differ-ce between the inhibitions brought about by Li and those by GSH.

Li, a vegetalizing agent, inhibits the animalization to such a degree that the undary between entoderm and ectoderm is forced so far in the opposite direction at the entoderm is hypertrophied. Li does not cure cell damages caused by the imalizing agents. On the contrary, the larvae suffer from injuries by this second eatment.

GSH, by no means a vegetalizing agent when tested on normal eggs, inhibits the imalization only to the degree of bringing about a normal animal-vegetal equi-rium. GSH improves the condition of the larvae by curing cell damages, caused the animalizing agents. The percentage of surviving larvae is thus increased by e GSH treatment.

It is thus probable that the inhibitions of the animalization, caused by Li and by SH, are founded on and followed by different reactions. Li and SCN may be sup-sed to interfere in mutually antagonistic chains of reactions, whereas GSH, and MA or thiomethylcytosine, may interfere as a couple of metabolite and antime-bolite in the same chain of reaction.

The animalizing effects of TMA and thiomethylcytosine should thus depend on a mpetition between the SH groups of the naturally occurring GSH and the added timetabolites, TMA and thiomethylcytosine. GSH alone did not bring about vege-lization here, but an addition of GSH to Li treated larvae enhanced the vegetalizing ect of this ion [9]. This does not imply any vegetalizing tendencies of GSH, but dicates that GSH is indispensable for the production of vegetal substances, an-gonizing the animal development. By treatment with TMA or thiomethylcytosine, e small amounts of natural GSH is, by competition, forced out of those positions, here the substance is essential for the production of these vegetal substances. By creasing the concentration of GSH, the competitive activity of the antimetabolites appreciably reduced, so that enough vegetal substances are produced to counter-lance the effect of the animal substances.

The author wishes to express his warm thanks to Professor J. Runnström and to . T. Gustafson for their interest in this investigation. My thanks are also due to e Authorities at Kristineberg's Zoological Station, where the experiments were rformed.

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THE STRUCTURE OF ADULT PERIPHERAL MYELINATED NERVE FIBERS AS REVEALED BY PHASE MICROSCOPY

W. G. ESMOND and A. SMITH

Departments of Surgery and Microbiology, University of Maryland School of Medicine, Baltimore, Md., U.S.A.

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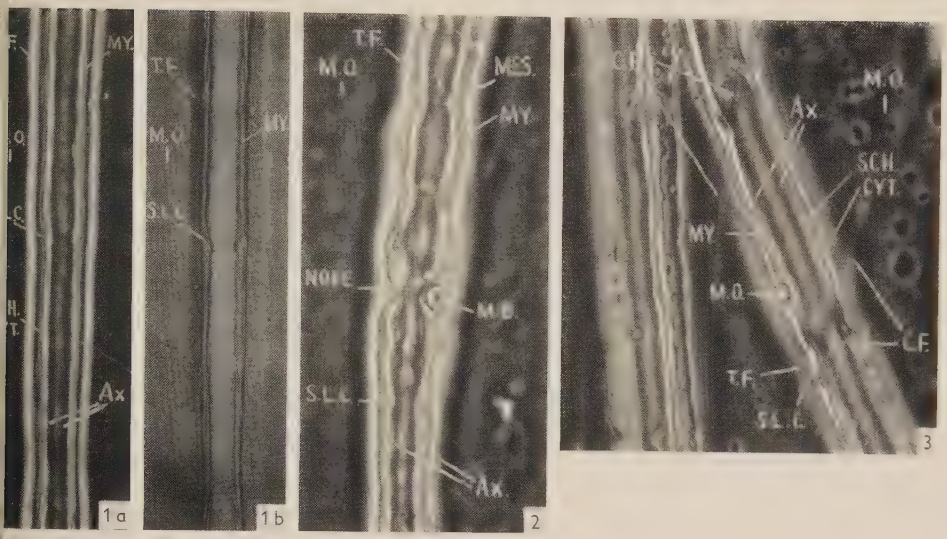
Recent electron micrograph studies of myelinated nerve fibers of chick embryos [3] and mice [4] have indicated that myelin is formed by the engulfment of the young axon by the Schwann cell followed by the formation of laminated myelin by a process of spiral wrapping of the Schwann cell around the axon. One of the most interesting features in many of these electron micrographs is the presence of considerable amounts of Schwann cell cytoplasm trapped between the multilayered myelin and the first layer of the Schwann cell against the axon termed the axolemma. In the earliest electron micrographs, it was not appreciated that this layer consists of cytoplasm but was attributed to shrinkage during fixation [3], but as more electron micrographs have been obtained showing considerable structural detail in this region there is now no doubt that many embryonic peripheral myelinated nerve fibers contain Schwann cell cytoplasm under the myelin layers between the axolemma itself and the laminated myelin. This annular layer is often greater in cross sectional area than the axon and is divided by the mesaxon into a spiral compartment which considering the three dimensional model, is continuous from the center of the fiber to the nodes of Ranvier. A two dimensional representation of these fibers seen in electron micrographs [4] appears to show segments of Schwann cell cytoplasm in multiple compartments bounded by the axolemma, mesaxons, and the laminated myelin layer, but a consideration of the spiral mode of myelogenesis indicates that these compartments are indeed one which appear to be multiple only because the electron micrographs represent a longitudinal section of a helical strucutre.

The presence of a significant cytoplasmic layer between axolemma and laminated myelin within the Schwann cell has not been previously recognized. In a series of papers dealing with the morphology of fresh adult myelinated nerve fibers of the frog under the light microscope [1, 2] de Rényi concluded that the axon was in direct contact with the myelin. De Rényi's observations are frequently cited in morphological studies of myelinated nerve fibers since they were done with great care on fresh material and are therefore thought to be free from artefacts that may be introduced by fixatives and stains. Electron micrographs of adult myelinated fibers usually show a considerable separation between the axon and the laminated myelin layer, but this separation has been considered to be artefact due to shrinkage based on de Rényi's observations of living fibers.

We have become interested in this problem in morphology because as it has been clearly shown that large amounts of Schwann cell cytoplasm may exist between axolemma and laminated myelin in embryonic fibers and its existence in any amount



g. 1, *a*.—Individual peripheral myelinated nerve fiber from sciatic nerve of the frog. Phase ntrast. Note the layer of Schwann cell cytoplasm between axon and myelin.

g. 1, *b*.—Individual peripheral myelinated nerve fiber from sciatic nerve of the frog. Identical ld as seen in *a*, but annulus has been removed from sub-stage condenser to eliminate phase ntrast. The layer of Schwann cell cytoplasm between axon myelin is no longer visible.

g. 2.—Individual peripheral myelinated nerve fiber from sciatic nerve of the frog showing a de of Ranvier. Phase contrast. The layer of Schwann cell cytoplasm between axon and myelin clearly seen.

g. 3.—Several peripheral myelinated nerve fibers from the sciatic nerve of the frog. Phase ntrast.

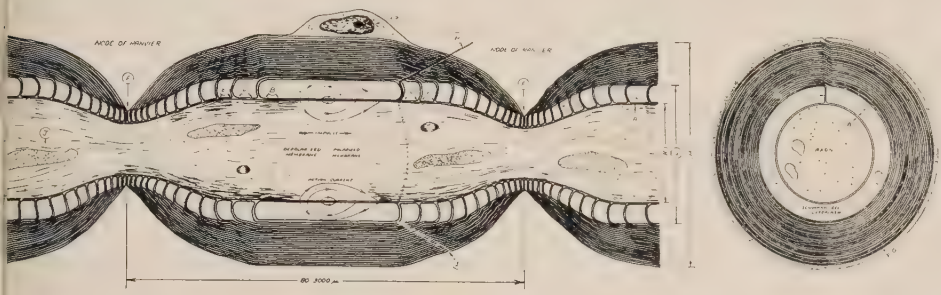
ey. AX, axon; MY, myelin; SCH. CYT., Schwann cell cytoplasm; S.L.C., Schmidt Lantermann eft; M.O., mineral oil; T.F., tissue fluid; MES., mesaxon; C.F., collagen fiber.

xceeding a layer of 200 300 Å thick is denied in adult fibers there may be some eriod in growth and development when this layer is gradually absorbed. On the her hand, it is also possible that the same relationship is carried into adult life, but ue to the methods used for observation, this fact may have escaped detection. If e system does indeed exist in adult fibers, it consists of two cytoplasms (i.e., xoplasm and Schwann cell cytoplasm) separated by an exceedingly delicate double embrane (i.e., the polarized membrane of the axis cylinder and the innermost wrap the Schwann cell around the axon which is termed the axolemma) only a few Å ick. Such a system could easily escape detection by the observer using the light icroscope. We decided to study the morphology of living myelinated nerve fibers the frog and the mouse since if the same morphology present in embryonic fibers uld be shown to be present in adult fibers, the study of fibers at different stages growth and development would be unnecessary. The phase microscope was chosen r this work since it is admirably suited for the detection of fluid compartments which ay vary slightly in refractive index.

Myelinated nerve fibers from the sciatic nerve of the frog and mouse were employed in this investigation. In the case of the frog, the nerve was promptly removed after pithing and placed on a microscope slide in frog Ringer's solution, and carefully dissected to liberate individual myelinated nerve fibers. The preparation was then covered with a coverslip and observed under dark phase contrast on the stage of a Bausch and Lomb phase microscope. The myelin tube under phase contrast appears as a bright wall about 15 per cent in thickness of the total diameter of the fiber. Myelin was easily identified by searching for the delicate lines indicating the clefts of Schmidt Lantermann. In the undamaged fibers which were chosen for study, these clefts are delicate lines crossing the myelin tube gradually and could be identified by noting the fact that they were symmetrical on both sides of the myelin tube. In all cases of undamaged fibers studies, the axon was clearly seen within the myelin tube separated from the compacted myelin layer by a clear bluish tinted cytoplasmic layer of considerable thickness. Under suitable viewing conditions, mesaxons could often be seen to cross the bluish tinted cytoplasmic layer and form a line on the axon as it wrapped around the axon. The presence of mesaxons in this layer positively identifies the layer as Schwann cell cytoplasm. The relative thickness of the layer between axon-axolemma membrane and the laminated myelin has averaged 10 per cent of the diameter of the fiber. When the annulus was removed from the sub stage condenser to eliminate phase contrast, the tubular axon promptly disappeared and the myelin tube appeared to be filled with homogeneous cytoplasm (Fig. 1 *a*, Fig. 1 *b*). We found as our study continued, that the dissection of individual fibers was facilitated by placing the nerve in a drop of mineral oil before dissection. Evaporation of tissue fluid is prevented and the individual fibers can be clearly seen in the mineral oil surrounded by their own tissue fluid (Figs. 1, 3). Experiments were performed with nerves prepared in this manner which indicated that these fibers were still capable of conducting impulses as evidenced by twitching of the attached muscle distal to the operated area when a stimulus was applied to the proximal cut end of the nerve. We have frequently found damaged fibers from which bare axons can be followed back to their junction with the myelin tube and can be clearly seen to be separated from the myelin tube by the cytoplasmic layer previously described. We have repeated these observations on numerous occasions with equally reproducible results and consider that we have conclusively demonstrated that the peri-axial Schwann cell cytoplasmic layer found in embryonic myelinated fibers is carried over intact into adult myelinated fibers. The cross sectional area of this cytoplasmic layer surrounding the axon is approximately equal to the cross sectional area of the axon itself and the function of this structure in the conduction of the nervous impulse must be considered. The saltatory theory of conduction in myelinated fibers, currently popular for several reasons among which is the previous failure to demonstrate an anatomical substrate for an external circuit for the continuous conduction of the nervous impulse within the myelin tube, must be reevaluated on the basis of these findings.

Fig. 4 illustrates the conception of one of us (W. G. E.) of the structure of adult myelinated nerve fibers in which our findings are correlated with electron micrographs published by other investigators. The layer of Schwann cell cytoplasm surrounding the axon is clearly pictured and appears to be divided, in this longitudinal section, into multiple compartments by the mesaxon. Actually, this compartment is a

ngle helical structure. The presence of a cytoplasmic layer equal in cross sectional ea to the axon, surrounding the axon, suggests that this structure may serve as t impedance matched external circuit for the continuous conduction of the nerve pulse within the myelin tube. Sodium ion is known to be essential in the medium rrounding axons for the formation and propagation of impulses and it is sug-sted by these findings, that Schwann cell cytoplasm may be an unusual cytoplasm



g. 4.—Diagrammatic representation of structure of adult peripheral myelinated nerve fiber.

y. F, node of Ranvier; I, Schmidt-Lantermann cleft; J, mitochondria; G, nucleus of Schwann ll; K, diameter of axon. L, Diameter of axon plus Schwann cell cytoplasmic layer; M, total di-meter of fiber; A, B, axon-axolemma membrane; and C, mesaxon; H collagen fiber.

ntaining large amounts of sodium ion in preference to potassium ion. The spiral ode of myelogenesis, first suggested by Geren, offers an interesting rational for the rmation of the Schmidt-Lantermann clefts. Collagen fibers may partially encircle e axon prior to myelin formation (Fig. 4, H). The enveloping Schwann cell may be able to push some of these fibers out of the way, and the enveloping lamina of e Schwann cell is cleft by the collagen fiber as rotation occurs, thus causing a fect in each myelin layer throughout 360° of rotation (Fig. 4, I).

Summary.—Phase microscopy of adult mouse and frog myelinated peripheral rve fibers clearly shows that a significant annular layer of Schwann cell cytoplasm rrounds the axon under the myelin and is approximately equal in volume to the oplasm itself. The possible significance of this layer in nervous impulse conduction discussed.

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INFLUENCE OF ESTRADIOL ON THE ULTRASTRUCTURE OF MOUSE UTERINE SURFACE EPITHELIUM

O. NILSSON

Department of Histology and Laboratory of Ultrastructure Research, Department of Anatomy, Karolinska Institutet, Stockholm, Sweden

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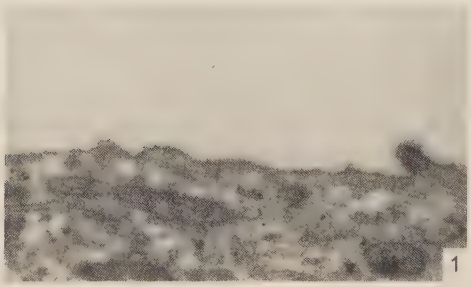
Administration of estrogens to rats gave among other effects an increase in the water and electrolyte content of the uterus [1, 2, 7]. A peak in water inbibition by the uterus of castrated rats was shown to occur within 4–6 hours after intravenous administration of estradiol [6]. An increase in the uptake of radioactive sodium and potassium over controls was found after the same interval in immature rats upon an intraperitoneal dose of estradiol [5].

This investigation was undertaken to determine if the shifts in uterine water and electrolytes were accompanied by changes in the fine structure of the epithelial cells.

Spayed mice of the C3H strain were used. The ultrastructure of the uterine epithelium was examined from spayed animals without extraneous estrogen and from spayed animals, sacrified at different intervals after an intravenous injection of 0.1 μg estradiol.

Electron micrographs of uterine epithelial cells from animals, sacrified 6 hours after estrogen administration, showed changes in the appearance of the luminal cell membranes and in the system of α-cytomembranes. In spayed animals, the cell surfaces possessed a few projections about 0.3 μ long (Fig. 1), but the uterine surface membranes of those animals that had been given estradiol showed a higher number of these cell projections (Fig. 2). A preliminary estimation showed that 2–3 times more projections pro square micron were found extending from the epithelial surfaces of uteri from estrogen-treated animals.



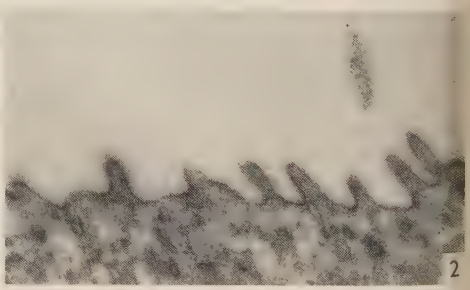


Fig. 1.—Luminal surface of uterine epithelium from spayed mouse. Magnification × 18,000.

Fig. 2.—Luminal surface of uterine epithelium from spayed mouse, sacrified 6 hours after an injection of estradiol. Magnification × 18,000.

The α-cytomembranes in the cytoplasm of spayed animals formed a system of small aces of different forms. Administration of estradiol caused an augmentation of both e number and size of the spaces.

The changes in the structure of the cell membranes and in the cytoplasm of the erine epithelial cells upon an injection of estradiol might be correlated with the ifts of uterine water and electrolytes, which were especially pronounced 4–6 hours ter the administration of the hormone. A similar variation in the ultrastructure of e cell membrane was described in the electric organ of the Egyptian catfish [3]. is variation also was suggested to be correlated with the shifts in water and elec-olytes during the different functional states of the organ.

The effects of estradiol on the uterine surface epithelium of mouse will be described ore fully in subsequent papers [4].

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CYTOPLASMIC NEUTRAL RED–RIBONUCLEIC ACID COMPONENT NOT INFLUENCING PROTEIN SYNTHESIS

W. S. MORGAN

James Homer Wright Pathology Laboratories, Massachusetts General Hospital, Boston, Mass., U.S.A.

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was shown earlier [9, 10] that if mice were injected subcutaneously with a pre-termined amount of the dye, neutral red, in solution, small reddish granules could on be observed microscopically in the cytoplasm of a variety of cells. It was later tablished [11] that the ability of a cell to take up neutral red —excluding cells of e reticulo-endothelial system —bore a direct relation to its basophilia and ribonu-ic acid (RNA) content. Accordingly, neutral red granule formation was greatest the pancreas and salivary gland. The neutral red granules themselves were baso-ilic, and this stainability was lost after treatment of sections with ribonuclease licating that they were made up at least partly by RNA. They also stained posi-ely by Baker's acid hematin method [1, 2] for phospholipid.

TABLE I. *Comparison of the pancreatic amylase content of mice feeding, fasted and given neutral red.*

Amylase method was that of Huggins and Russell [7]. Neutral Red was obtained from the Allied Chemical and Dye Corporation, New York, N.Y., as Neutral Red Chloride, certified (Tolylene Red), Color Index No. 825. It was made up as a 1 per cent solution in distilled water. Each subgroup was made up of 4 adult male mice weighing about 25 g. Equal portions of the four pancreases were combined totalling 0.4 g wet weight and this homogenized in 8 ml 0.25 *M* sucrose. The pH of the homogenates was 6.8. The zymogen granule sediment was resuspended in 5 ml 0.25 *M* sucrose for optical density reading.

Group	Procedure	Amylase level of whole homogenate (units)	Optical density of zymogen granule suspension
A_1	Feeding mice killed 7:15 p.m.	35,000	0.129
A_2		43,000	0.213
B_1	Feeding mice given 0.8 ml distilled water subcutaneously 10 a.m.; fasted until 7 p.m.	40,800	0.171
B_2		39,000	0.180
C_1	Feeding mice given 0.8 ml 1 % Neutral Red subcutaneously 9:45 a.m.; fasted until 6:45 p.m.	59,000	0.387
C_2		55,000	0.249

In view of these findings it was wondered what effect the formation of this dye-RNA complex had on protein synthesis. This was investigated by a study of amylase formation in the pancreas and by the measurement of uptake of radioactive leucine by Ehrlich mouse ascites tumor cell protein. In the pancreatic studies, the classic method of using pilocarpine to deplete the gland of zymogen granules was not employed because of the concurrent effects of this drug on the adrenal gland, liver glycogen and blood sugar level [12]. The pancreas in the mouse has been shown to secrete continuously [6]. Therefore, it could be expected that any substance that inhibited protein synthesis in the pancreas would eventually result in a low amylase content of the gland. This possibility was tested by comparing the pancreatic amylase level of a control group of feeding mice with that of a group given neutral red and killed 9 hours later. During the period chosen for the experiment neutral red granule formation is marked. Since animals do not eat for several hours after being given the dye, it was believed advisable to include an additional group that was fasted. The

riods included in the experiment are within the replacement rate of the pancreas nce it has been found that mice given pilocarpine with depletion of zymogen gran- es regain normal amylase levels in 5 to 7 hours [13]. Table I demonstrates that e amylase content of the pancreas of mice given neutral red is not lower than that the control feeding or fasted animals. On the contrary there seems to have been cention of the enzyme; the explanation for this is not clear at the present time. Though neutral red did not affect the amylase content in the pancreas it might ve interfered with the elaboration of the mature zymogen granule such as blocking "finishing" process. Attempts to assess the zymogen granule content by histological eparations were not satisfactory. It was possible to determine the concentration zymogen granules by the method of Hokin [5] in which they were isolated from mogenates by differential centrifugation in 0.25 *M* sucrose, resuspended in sucrose d their turbidity compared in the spectrophotometer employing a 650 mμ filter. ne last column of Table I shows that the zymogen granules rather than being de- eased in number were actually increased in animals given the dye. This finding is line with the high amylase content in these same specimens. These results indicate at the presence of the dye bound to an RNA component in the pancreas exocrine ll does not inhibit either the formation of the digestive enzymes or the maturation the zymogen granules in which the enzymes are normally contained.

The second test of the effect of the dye on protein metabolism was made using e method of Littlefield and Keller [8] in assessing the uptake of radioactive leucine o proteins of ascites cells. Mouse ascites tumor cells contain a relatively large nount of RNA [3]. If an animal to which tumor cells have previously been trans- anted is given an intraperitoneal injection of neutral red, the ascites cells soon ow prominent neutral red granule formation. These usually are concentrated near e nuclear cleft where the cytoplasm is most dense as shown by Selby *et al.* [16] electron micrographs. When unstained cells are examined in the light microscope is area is rather homogeneously finely granular. This would seem to rule out the ssibility that the neutral red granules represent pre-existing vacuoles which become ained by the dye. It might be mentioned, however, that the dye granules remain screte and in this regard differ from those of the pancreas which soon take the form aggregates. Another difference is the observation that in the fixed sections of cites cells the neutral red granules do not appear as basophilic or pyroninophilic dies as they do in pancreas. In view of their morphology, however, the assumption s been made in these studies that they do represent the counterpart of the granules nd to contain RNA in the pancreas. A possible explanation of this discrepancy the fact that the RNA content of the acinar cells is much greater permitting pro- ction of a granule that is microscopic in size; or it may have to do with some struc- ral difference in the respective particles taking up the dye. Male mice of the Web- r strain weighing 26–28 g were injected intraperitoneally with 0.5 ml ascites fluid m a previously inoculated animal. Nine days later 10 such mice weighing now –42 g were divided into two groups of five. Mice of group A, the control group, re given 1.3 ml distilled water intraperitoneally; those of group B, the test group, re given 1.3 ml of a 1 per cent solution of neutral red made up in distilled water. pproximately 1½ hours later, the mice were killed and the ascites fluid removed. As seen in Table II the formation of the neutral red granules in the mouse ascites ll does not significantly influence the rate of amino acid incorporation into protein.

TABLE II. *Comparison of incorporation of* L-*leucine-1-*C^{14} *into protein of ascites tumor cells.*

The complete system contained in a final volume of 2.0 ml 1.6 ml ascites fluid and a final concentration of 0.01 *M* L-leucine-1-C^{14}, containing 17,500 c.p.m./μM, 0.02 *M*. Tris buffer (pH 7.6), and 0.04 *M* glucose. Incubated 15 min. at 37°C in air. We can calculate [5] that the average rate of labelling of the protein was about 0.81 per cent per hour in the controls and 0.68 per cent per hour in the neutral red-treated animals.

Control (c.p.m. per mg protein)	Neutral Red (c.p.m. per mg protein)
19.3	30.9
22.8	27.2
28.7	15.4
38.3	18.2
25.6	21.4

These studies support the conclusion that the binding of this particular cytoplasmic RNA by neutral red does not affect protein synthesis. This conclusion makes possible the further suggestion that the RNA component having an affinity for neutral red is not normally involved in any operation directly related to the elaboration of the spherical bodies containing the pancreatic enzymes. It seems most unlikely that when this component is in the form of neutral red granules it can simultaneously carry on some other function. The same can be said of its involvement in amino acid incorporation into ascites cell proteins.

Cytologically the neutral red granules in the pancreas acinar cell occupy a characteristic position between the lowest level of the zymogen granules and the upper level of the nucleus. Therefore, it would appear that the RNA of the neutral red granule is not the RNA of the endoplastic reticulum [14] which is more concentrated in the basal portion of the cell. In view of these observations, it seems reasonable to suggest that the neutral red labels another RNA component of unknown function, and indications are that this is normally beyond the resolution of the light microscope but becomes microscopic by its affinity for the dye and subsequent swelling. It is possible that this cytoplasmic component involves the Golgi element. This conclusion is supported by our previous finding that the neutral red granules stain positively when the classical Golgi methods are applied [10]. Further support is provided by the presence of RNA in the neutral red granules and the fact that Schneider and Kuff [15] found a high concentration of RNA in their Golgi fraction isolated from rat epididymis. If it is true that the neutral red granules represent dye-labelled Golgi substance, the data presented above in regard to the pancreas imply that the Golgi substance is not directly involved in the formation of the secretion granules, i.e., the latter are not formed within the Golgi vesicles as previously suggested by some [4]. Zymogen granules have never been observed issuing from the neutral red granules and the two occupy different parts of the cell.

The ascites tumor cells contain a relatively large amount of cytoplasmic ribonucleic id and are rather deficient in the membranous phospholipid component of the endo- asmic reticulum [3]. This observation coupled with the fact that neutral red granule rmation is very prominent suggests that the ribonucleic acid plays a greater role their development than the phospholipid.

Although Weiss [17] has made a study with the electron microscope of the pancreas mice after administration of neutral red, our knowledge of the fine structure of he neutral red granule is not yet sufficiently certain to permit a conclusive state- ent as to whether the granule represents a single particulate component having affinity for the dye or whether it reflects a combination of cytoplasmic elements acting in response to neutral red.

It was mentioned earlier that the ability to form neutral red granules is greatest cells having a high RNA content and as is well known these are the cells most ac- vely engaged in protein metabolism. The results of the experiments described here ave indicated that this RNA component apparently normally is not involved either the amino acid incorporation or in any other phase in the elaboration of the mature nzyme granule of the pancreas. Therefore, it remains to be learned what the function this RNA-containing component is, and, if it does not contribute in some way to rotein synthesis, why it is so prominent in cells in which this is a major function. his question takes on added interest if it is true that neutral red labels the Golgi ements of the exocrine pancreas for the so called Golgi apparatus has long been be- eved to be much involved in the formation of secretion granules.

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MYELIN FIGURES OCCURRING IN THE PERIPHERAL NERVE BUNDLE OF HUMAN SKIN

A. CHARLES

Departments of Dermatology and Biomolecular Structure, The University, Leeds, England

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Stoeckenius [4] first reported the laminated structure of tissue mast cell granules, which had also been observed in these laboratories. Subsequently Policard *et al.* [2] and Stoeckenius [5] reported the laminated construction of myelinated figures arising from red blood corpuscles after their ingestion by phagocytizing macrophages. The present communication shows that a similar structure occurs, or can arise, in the endoneurium of the peripheral nerve bundle of human skin.

Material was obtained from the scalp of a male aged 57 years under general anaesthesia for craniotomy and removal of tumour. After cutting into suitably sized cubes the material was fixed in 1 per cent osmium tetroxide in veronal buffer pH 7.4 [1]. Fixation commenced about ten minutes after excision. Subsequent treatment followed the usual routine methods, and terminated in the embedding of the material by polymerization in methacrylate monomer at 45°C.

In Fig. 1 the relationship of the myelin figures (boxed) to one of the nerve myelin sheaths (*m*) is shown, while the inset Fig. 2 is an enlargement of the boxed region.

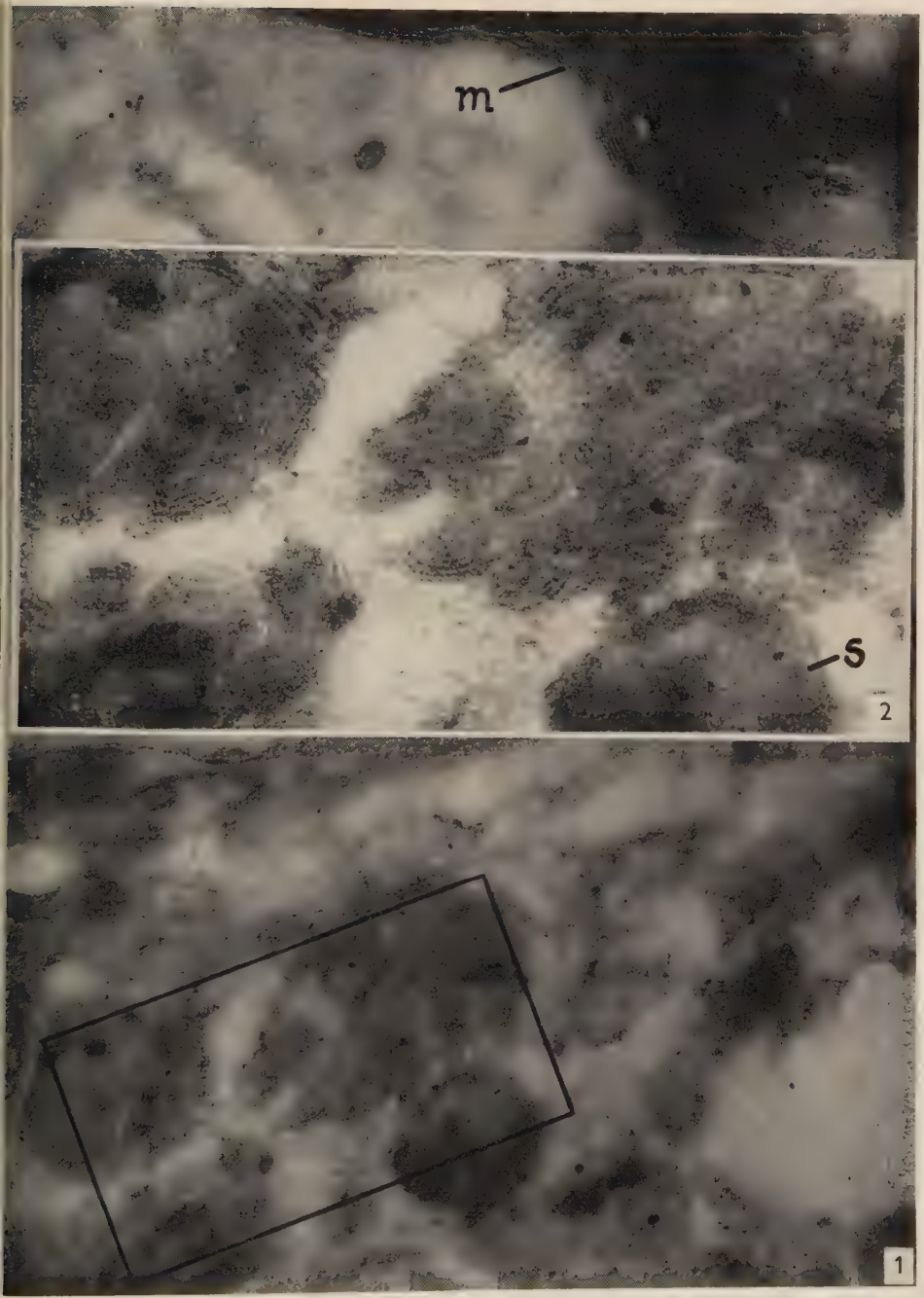
We see here the same multi-centric laminated whorls as were described by Stoeckenius [5]. In some cases, indicated by (*s*) in Fig. 2, the laminations are replaced by a homogenous material of the same density, due presumably to the laminations being seen in surface section.

The dimensions of the laminations in the endoneurium agree approximately with those given by Stoeckenius for the mast cell granules, which my own observations confirm, the laminae being about 100 Å part and about 50 Å thick, with a clearer region of about 50 Å between. These dimensions differ somewhat from those found for the myelin figures of the phagocytized red blood cell which themselves show appreciable variation, so that it may be difficult to gain an idea of the nature of the lipid from such measurements.

A 100 Å period seems true also for the myelin sheath of the medullated nerve, but the denser lamellae in this case are much more sharply defined than in the endoneurial structures, and the latter do not show the 50 Å period [3] faintly observable in the myelin sheath when Fig. 1 is further enlarged.

Fig. 1.—Section of a peripheral nerve bundle showing the relationship between one of the myelinated nerve sheaths (*m*) and the myelin figures (boxed) found in the endoneurium. ×60,000.

Fig. 2.—Enlargement of the myelin figures in the boxed area of Fig. 1, showing the multi-centric laminated structure. In some cases (*s*) the granules have a homogenous structure which may partly be due to the laminations being seen in surface section and possibly also to the presence of non-laminated material. ×110,000.


m
S
2
1

Originally I was inclined to draw comparison between the laminated structures in the endoneurium and the laminations of the mast cell granule. Such a well-organized structure, I thought, associated as it was in the mast cell with secretory products, must surely have a function. But the publications [2, 5] mentioned above make it fairly clear that this assumption was not justified; the laminations are a result of the physico-chemical behaviour of various lipids, the molecular mechanism of which is a point of interest [2, 5].

I am pleased to be able to express my great indebtedness to Mr. A. E. Wall, Neurosurgeon at the Leeds General Infirmary, for his greatly valued co-operation in providing material.

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VARIATION OF DESOXYRIBONUCLEIC ACID CONTENT OF SPECIFIC CHROMOSOME REGIONS[1]

H. F. STICH and J. M. NAYLOR

Saskatchewan Research Unit of the National Cancer Institute of Canada, Saskatoon, Canada, and Field Husbandry Department, University of Saskatchewan, Saskatoon, Canada

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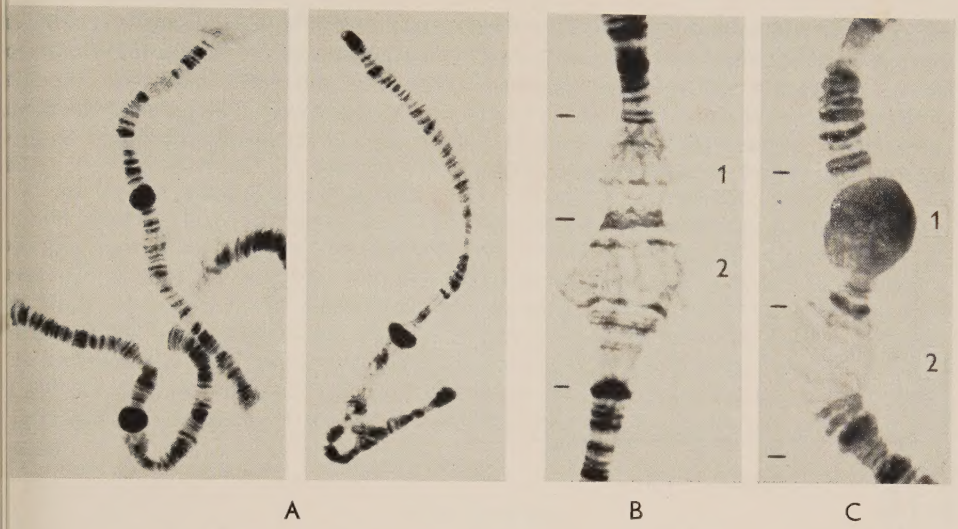
An exact correlation between the DNA content and the chromosome set was originally postulated by Boivin *et al.* [4], Mirsky and Ris [9], and has been confirmed by numerous microphotometric measurements on various tissues of animals and plants [15, 17]. The few published contradictory results that indicated variation in the amount of DNA in diploid nuclei were based on the one wave-length method; these have been questioned, and attributed to technical errors [15]. However, there are cases described where DNA containing corpuscles are extruded from the chromosomes or nuclei [2, 14] and these do not agree with the established view. Similarly, certain factors such as temperature apparently induce departures from DNA constancy in some plant nuclei [8]. The present study has been undertaken to investigate this problem of constancy or inconstancy of the DNA-content per chromosome set.

[1] Acknowledgements are given to Dr. H. Bauer for suggestion of the material used in this study and to Dr. J. Rempel for assistance in obtaining the larvae. Following the completion of this work, similar experimental results and conclusions, based on U.V. absorption measurements with salivary chromosomes of *Rhynchosciara* have been reported by Rudkin and Corlette (*Proc. Nat. Acad. Sci.* **43**, 964 (1957). We are grateful to Drs. J. Schultz and G. T. Rudkin for calling our attention to this report.



ig. 1.—The three long salivary gland chromosomes of Glyptotendipes after accumulation of)NA in the medium puffed regions (*A*). Larger magnification of adjacent puffed regions before 3) and after incubation of the larvae at 18°C (*C*). The corresponding bands are marked. eulgen-reaction.

The salivary gland chromosomes of Glyptotendipes (*Chironomidae*, *Diptera*) were hosen as the subject, since their great length on squash preparation permitted the ieasurement of DNA in single bands or puffed regions which have been analyzed ı detail by Bauer [1]. Of specific interest in regard to the above mentioned problem ere the puffed regions located approximately in the middle of each of the three)ng chromosomes. One of these "puffs" in each chromosome develops a strongly eulgen-positive globule during the later stages of the fourth larval instar period ig. 1*a*). Other centrally located puffed regions of the same chromosome remain ithout any visible morphological alterations. Formation of the DNA-rich globules prevented when the larvae are held at low temperatures (6–8°C), but occurs within –3 days after transfer to 18°C. These conditions allowed the determination of the)NA content in a single puffed region of the chromosome before and after accumula- on of the Feulgen-positive globule, and the comparison of the relative changes oc- urring in different puffed regions on the same chromosome.

The chromosomes were prepared as follows: the salivary glands were extirpated om the fourth instar larvae; fixed in alcohol: acetic acid (3:1) for 20 minutes at)om temperature; transferred to 45 per cent acetic acid for 10 minutes; squashed n a slide and placed into 95 per cent alcohol (two changes) at 4°C for 16–20 hours; ydrolysed in 1 *N* HCl at 60°C for 10 minutes; stained with the Feulgen reagent for .5 hours; washed in SO_2 water (three changes) and mounted in immersion oil of n_D 1.515. Microphotometric DNA measurements in specific chromosome segments ere performed by the two-wavelength method of Patau [11] and Ornstein [10] sing the correction factors of Swift [16]. Because of their great length, entire chromo-

somes could not be included within a single measured field. Consequently, the DNA content of whole chromosomes was found by summation of the values for small segments. Reliability of DNA determination with the apparatus used was checked in a series of measurements on nuclei of known DNA content. The ratio of the mean values for telophase (2C) and prophase (4C) nuclei deviates slightly more than 2 per cent from the 1:2 ratio for these nuclei.

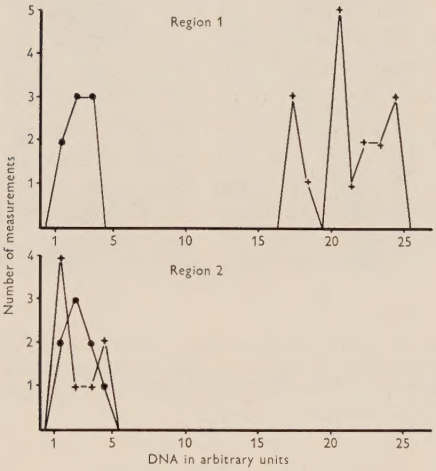


Fig. 2.—Changes in DNA content in regions 1 and 2 of the same chromsomes in 8°C (= •) and 18°C (= +).

The results are shown in Fig. 1*b* and *c*, which illustrates the morphological changes of one of the three long chromosomes and in Fig. 2, which demonstrates the DNA content in the two adjoining puffed regions before and after incubation at 18°C.

The 8-fold increase in DNA occurring on puffed region 1 cannot be attributed to alterations of the degree of polyteny or to incorporations of adjoining chromosome parts into the DNA globule. The first possibility can be excluded because the second puffed region did not show an upward shift in DNA content during the 3 day period of the experiment and the second because the puffed region and the globule occupy the same locus on the chromosome as can be checked on the band pattern. The conclusion that the DNA content of specific parts of the chromosome change independently seems, therefore, to be justified. This inconstancy of the DNA content becomes still more evident if the larvae are returned to a temperature of 6–8°C. The accumulated DNA then disappears gradually. The DNA loss was associated with a swelling and disintegration of the globule which prevented reliable microphotometric measurement. These results resemble the findings of Breuer and Pavan [5], who found an increase and decrease of Feulgen-positive material on specific regions on *Rhynchosciara*, although they have not presented any quantitative data. On the other hand the behaviour of the second puffed region on *Glyptotendipes* is similar to that in *Drosophila* salivary gland chromosomes, where the nucleic acid content does not change during the puffing of this region [12].

This DNA variation might be of some interest in regard to the problem of gene action. The idea is attractive that DNA molecules which are produced in excess on

e chromosome loci diffuse into the cytoplasm transmitting gene information. Al-
ough this suggestion remains purely hypothetical, it would be in good agreement
th the facts that the DNA molecules are highly specific components of the genes
d possess the ability to transfer specific characteristics [18]. The exceptionally
gh local incorporation of thymidine in similar regions in salivary gland chromo-
nes of *Rhynchosciara* [7] supports the above conclusion. Such an idea, of course,
based on the assumption that the DNA content is *not* constant, which must be
oved to be a general phenomenon. In this connection it is important to stress the fact
at the amount of DNA which exerts the variations described above amount to only
out 10 per cent of the DNA of the entire chromosome. This means that in measuring
e whole chromosome or nucleus DNA constancy might be postulated erroneously,
ce this DNA variation could scarcely be distinguished from technical error. Taking
o account the data presented here, the extrusion of DNA from the chromosomes
14], and the evidence of limited DNA turnover [3] it seems that the DNA content
ght not be absolutely constant per chromosome (or chromatid) set as widely pro-
sed.

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Experimental Cell Research SUPPLEMENT 4

CYTOCHEMICAL METHODS WITH QUANTITATIVE AIMS

BIOPHYSICAL AND BIOCHEMICAL APPROACHES

Proceedings of the Symposium held September 27–29, 1956, by the Institute for Medical Cell Research and Genetics, Karolinska Institutet, Stockholm, Sweden

Edited by Bo Lindström, *Karolinska Institutet, Stockholm, Sweden*
and R. Brown, *Oxford University, Oxford, England*

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Experimental Cell Research SUPPLEMENT 5

SUBMICROSCOPIC ORGANIZATION AND FUNCTION OF NERVE CELLS

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